

Troubles of a divided island

Irish politicians have not risen to the challenge of charting a future for Ireland. The time has come for the intellectual community to take charge of events.

FOR the umpteenth time in 350 years, a British government is in a pickle about Ireland. The latest conundrum thrown at Westminster is last week's report of the New Ireland Forum, the rump of what was conceived in 1982, by the British and Irish governments, as a means of shaming Irish politicians into some kind of agreement among themselves about the future constitution of Ireland and in particular that of Ulster, the still-British north-east corner of what is otherwise Irish Ireland. After more than fifteen years of urban and rural violence by an organization illegal on both sides of the border, the hope behind the forum was that Irish politicians would be able to conjure from their despair some goal at which, collectively, to aim.

Hope was from the outset dimmed by the refusal of the political parties in Ulster bent on preserving the link with Great Britain to participate in the proceedings of the forum. Now, hope has predictably been dashed by what the forum has to say — that the ideal solution for Ireland is that the two parts should be one, but that partial (or temporary?) solutions might be found in a federal constitution or in an arrangement under which the British and Irish governments would have shared responsibility for the administration of Ulster. The result will be that attitudes on each side of the border will be further hardened: the Protestant part of Ulster will be confirmed in its belief that any change must be unwelcome, the Roman Catholic half and the Republic to the south persuaded that republican nationalism is respectable. And the gunmen will say that their actions have been justified.

Not unique

Readers, quite properly, do not give journals such as this a licence to take on issues complicated, like that in Ulster, by political and sectarian problems. Nor is one sought. But it is noticeable, and a little strange, that most of the opinions offered on the problem of Ulster start from the assumption that the problem is unique — and uniquely Irish. Can that be so? Has not Belgium been riven for decades by similarly founded if less violent tensions between linguistic and religious communities? What about the problem of the Sikhs in India's Punjab? Or the Tamil community of Sri Lanka? And the East African community of Indian origin, mostly expelled in the past fifteen years to the great profit of the English, the nation of shopkeepers which now at least, has efficient shopkeepers? Ireland cannot be as different as it seems. Is it too much to ask that the politicians always earnestly seeking solutions on a different tack should look at the sorry record of what has happened elsewhere?

What might be learned? Two principles emerge from the few illustrations of how communal divisions can be successfully accommodated (Belgium now, Switzerland a few centuries ago). First, palpable injustice sensed by the members of one community must be removed, if necessary by law. Second, all those concerned must have the patience to persist with the remedies they consider wise. The first condition is well on the way to being met in Ulster. The trouble is that the two governments concerned have not been able to wait long enough to allow even sensible reforms to work their way through the societies affected.

A decade ago, in Ulster, when Mr John DeLorean set up shop to manufacture sports cars for a non-existent market, the British government was wedded to the notion that industrial investment would eventually heal the wounds in Ulster. Four years ago, both

the British and the Irish governments were earnestly seeking bridges to build between north and south — increasing the exchange of electricity, for example, joint planning on environmental matters and the exchange of police intelligence (about the gunmen operating on each side of the border). More recently, with the ins and outs of governments in the Republic, attention seems to have shifted to the search for constitutional innovations.

For the intellectual communities both of Britain and Ireland, the consequences of these shifting strategies are, or should be, intolerable. In many ways, the two countries are virtually indistinguishable. They are not even divided from each other by language — Ireland is a disproportionately rich contributor to English literature (as well as to British television). Are there not ways in which these intelligent people could, without waiting for the politicians, create bridges that would last?

Education

The obvious place to start is in higher education. As things are, there is a trickle of students from the Republic to British universities, where they are treated on an equal footing with students from elsewhere in the European Community — they pay the same low fees as British students, but receive no help with maintenance from either government. There is a more substantial movement of students from Ulster to the Republic, mostly of Roman Catholics seeking an education at the National University of Ireland (a federal institution with separate institutions in Dublin, Cork and Galway). Why, to begin with, should not those who manage the two university systems work out an equitable basis for running them as if they were one? There is every reason to expect that if the British and Irish universities were satisfied with some joint plan of action, the two governments would jump at the chance of meeting the trivial costs entailed. And the same is true of graduate education — the British research councils, for a start, should go back on their decision of a few years ago that they would not thereafter treat graduate students from the Republic on an equal footing with their British equivalents.

There is no obvious end to the opportunities which thus suggest themselves. Why maintain in each of these two similar countries quite separate organizations for supporting agricultural research (on which both halves of Ireland are largely dependent?) Why have two weather forecasting services, one of which (the British) solemnly puts out daily weather forecasts from which projections for the area of the Republic are solemnly omitted? And since there are no restrictions on the movement of people between Britain and Ireland, why not go the whole hog and regard the two research enterprises as parts of the same whole?

The obvious objection, that such arrangements would somehow diminish the national identity of the two partners, cuts no ice, while the observation that the forum's preferred solution is a united Ireland is irrelevant. Fears that, in such arrangements, one partner or the other would win unfair advantages, financial or otherwise, are more real but should not be beyond the wit of intelligent people who speak the same language. The objection that people whose chief interest is not politics but education and research should be indifferent to political opportunities is often heard, and might in normal circumstances be valid. But these, as the weeks ahead will show, are not normal but desperate times. □



Teaching generality

A proposal to broaden the British school curriculum is also a chance for the university system.

FIFTEEN years ago, the British educational establishment was bitterly divided by a proposal for the reform of school-leaving examinations. Recognizing that students planning to stay on in higher education were too often required to rehearse in advance the studies they would later follow at university, and that the consequence was often a lopsided education, many school teachers and some university teachers took up the cause of a broadening pattern of school-leaving examinations under the banner N (for "normal") and F (for "further"). The idea was that instead of spending their last two years of secondary education following courses as narrow in pattern as chemistry, physics and mathematics, or even Latin, Greek and ancient history, students would be able to study more broadly, perhaps bridging the longstanding gap between the physical and biological sciences or even acquiring some skill with foreign languages.

In the event, years of planning came to nothing, chiefly because of the universities' fears that their students would be too ill-equipped with specialist knowledge to gallop through the standard three-year degree course. Hence the surprise that Sir Keith Joseph, the Secretary of State for Education and Science, should last week have floated a rather similar notion, for school-leaving examinations called A (for "advanced") and S (for "supplementary").

The fierce arguments of the early 1970s are unlikely on this occasion to be resuscitated. In the interval, most of those concerned have been convinced that the peculiarly English pattern of secondary education to which intending university students are subjected is indeed too specialized. Secondary schools, increasingly concerned with the needs of students for whom there will be no room at university, have come to appreciate the absurdity of simulating for their academically-inclined students teaching which is more appropriate for the introductory part of a university course, while many of the students concerned have chosen to opt out, quitting their secondary schools in favour of other establishments with a more adult flavour, called colleges of higher education.

Scottish educationalists have been amused by this change of heart in the rest of the United Kingdom, knowing that they have for the past century operated a perfectly sound educational system not flawed by early and narrow specialization (but requiring that students should spend four years acquiring a first degree). One of the reasons why English universities have come round to the same way of thinking is the simple arithmetical calculation that the reduction in the scale of their activities decreed by the British Government would be neatly offset by the replacement of the most common pattern of three-year degree courses with the four-year pattern which is universal in Scotland (not to mention the United States and most places in Western Europe). This is what many universities have been telling the University Grants Committee in the past few weeks. The government's proposals on school-leaving examinations is in this connection a kind of advance counter-proposal — generality without the extra cost of longer university courses.

The danger now is that the discussion of the new examinations will be conducted on too narrow a front. The ideal is that it should be informed by a proper understanding of what secondary schooling is for. For too long (except in Scotland), British schools have been hamstrung by the need to cater both for the generality of their students and for the needs of those with academic inclinations. The system worked well enough, but inequitably, when the majority of students were invited to leave school at a tender age. Now that people must stay at school at least until sixteen, and while the shortage of jobs in the real world implies that young people stay on at school for longer than in the old days, the only respectable goal of secondary education is what it should have been in the first place — the provision for young people of an intellectual foundation on which adult skills may be founded.

British higher education, and the universities in particular, have a more awkward problem to solve. In 1970, it would have been possible to arrange for a coordinated change in the division of responsibility between the schools and higher education; now the schools are changing anyway, and new examinations will speed the process along. This time, however, the universities will have less influence than they might have had, and will lack the resources necessary to extend the bulk of their undergraduate teaching from three years to four. The way out of this dilemma is simple enough — the University Grants Committee, nearing the point at which it will have to decide how to shape the future of the British university system, should encourage one or two of them to become four-year institutions that select students a year earlier than now and possibly by means of criteria different even from the new school-leaving examinations. The system is much in need of diversity. This is merely one of the several ways in which it should be provided. □

Too many paymasters

The British Government's sale of its telecommunications monopoly highlights conflicting interests.

How do you best arrange to sell a monopoly business as a going concern? By doing everything you can to ensure the monopoly is intact, for then the bidders will pay a higher price. That is plainly the thinking underlying the British Government's announcement last week of the terms on which it will sell at least 51 per cent of its holding in the telecommunications service now called British Telecom. The intention is that the service will be converted into a public company of the ordinary kind, and that shares will then be sold to whoever will buy them at a price still to be decided. Most probably, all this will happen in the autumn of this year. For political reasons, especially so as to demonstrate that its policy of shedding nationalized industries is justifiable, the government is anxious that the sale of shares shall be a "success" — which will probably mean that the price will be pitched low, or "generously" as the stockbrokers say. Even so, the hope is that something in excess of £3,000 million will help to swell the British Government's income in the current year.

This irreversible step is the last in a series of swings of policy on telecommunications in the past five years, although its roots go back to the time when, in the late 1960s, Mr Tony Benn (as he is now) decided that for administrative purposes, the British telecommunications service should be managed as if it were a private business. By the time of the present government, now just five years old, it had been concluded that the public monopoly's insistence on being the sole supplier of terminal equipment should be ended, with the result that there has since been a slow widening of the equipment available for use with telephone lines. At one time, the government was also inclined to think that the best way of bringing the monopoly to heel would be to license competitors, and one of these (called Mercury) now actually exists. But the view that British Telecom should be sold on the stock exchange has put a stop to such diversions, while the disputes that persist between the monopoly and private companies supplying radio-paging and cellular radio services are unlikely to be settled until after the monopoly has been sold.

The pity is that there has never been a convincing case that selling British Telecom as a going concern would better serve the public interest than, say, breaking it into smaller pieces (as in the United States) or reducing its activities to the installation and maintenance of a network that people could then use as they chose (for a fair price). Nor, yet, has there been an informed debate about the terms of the licence under which the monopoly will be allowed to operate. Even the government's undertaking that for the next five years, the charge made by the monopoly for its services should be increased each year by less than the inflation rate less three per cent is meaningless: the government itself has been compelling British Telecom to finance its development costs from telephone charges when a public company would have been borrowing from the banks. □

Reactors for China

US agreement signed, not sealed

Washington

PRESIDENT Ronald Reagan's success in negotiating a nuclear sales agreement with the government of the People's Republic of China has lifted the spirits of the United States' embattled nuclear power industry. But its euphoria may be premature. For one thing, it is far from clear just how many US reactors the Chinese will eventually purchase. For another, the agreement has yet to be finalized and will face serious hurdles in Congress.

Before Congress gets a chance to see the small print of the agreement initiated by President Reagan in Beijing, the document must be cleared by a number of federal agencies, notably the Nuclear Regulatory Commission and the Arms Control and Disarmament Agency. Their job is to ensure that the agreement complies with the law on nuclear transfers enshrined in the Atomic Energy Act and the associated Non-Proliferation Act. But there is little question of either agency objecting to the agreement, which is the fruit of six rounds of talks held over more than two years. A senior administration official insisted last week that the agreement complied fully with both laws.

The agreement will have a much more difficult time in Congress, however, where it must remain for 60 consecutive legislative days before it can take effect. That means that even if it enjoys an untroubled passage through the House of Representatives and the Senate, it cannot come into force until the end of the summer. An untroubled passage is unlikely. The agreement will face considerable opposition from an unusual alliance forged between congressmen who worry about nuclear proliferation and those who object on ideological grounds to any deal with communist China.

One reason for opposition to the agreement is China's ambiguous record on proliferation. Washington's Nuclear Control Institute has been noisily reminding Congress that China is one of only two nuclear power states (the other is France) not to have signed the Non-Proliferation Treaty. The institute has also complained of reports that China has helped Pakistan's bomb programme, and provided enriched uranium to South Africa and heavy water to Argentina.

Paul Leventhal, the institute's president, argues that because of China's symbolic importance in the less developed countries, the United States should seek a model agreement rather than content itself with obtaining minimum compliance with US law. A "good" agreement, the institute maintains, should include a written guarantee from the Chinese that they will

not help other nations to acquire nuclear weapons, and a firm commitment to use its civilian nuclear power programme for strictly non-military purposes. China should also agree to prior US consent to the separation of plutonium from US fuel or from any fuel used in reactors supplied from the United States.

Administration officials have made it plain that China has indeed agreed to seek prior US consent, one of the thorniest requirements of the Atomic Energy Act and one that had threatened to scotch the agreement until the very last moment (see *Nature* 19 April, p.677). It has also promised not to help other countries to manufacture nuclear weapons and to apply International Atomic Energy Agency safeguards to its own nuclear exports. But many in Congress are concerned by administration hints that agreement on the prior consent issue had been reached through "clever" drafting designed to appease Chinese national sensibilities.

The degree of political opposition will depend largely on what is contained in the full text of the agreement initialled in Beijing. Under present law, Congress

German deal too

THE West German cabinet last week approved an agreement with the People's Republic of China for the peaceful application of nuclear energy. The agreement, signed formally on 9 May, originates in a Chinese initiative of December 1982. It covers research, development, safety and planning as well as the construction of power plants and research installations. West Germany is acting in accordance with its membership of Euratom and the European Economic Community and as a signatory of the convention on non-proliferation. China, however, is not yet a member of the International Atomic Energy Agency and there will therefore be no outside control to ensure that it keeps to the terms of non-proliferation and that neither technology nor materials are diverted into military channels.

Sarah Tooze

could not block the agreement without enacting legislation, although an amendment to the Export Administration Act could require Congressional approval for nuclear transfers. For the US nuclear power industry, however, the principal uncertainties may reside in Beijing rather than Washington. The Chinese are expected to approach the commercial negotiations with the same shrewd patience that they have shown politically.

Peter David

Poland

Jaruzelski urges discipline

MORE spending on science from 1986 onwards, increased cooperation with scientists in capitalist countries and annual meetings between leading scientists and politicians are among General Jaruzelski's recommendations for the development of Polish science. Addressing an eve-of-Mayday meeting at the Staszic Palace in Warsaw, the headquarters of the Polish Academy of Sciences, Jaruzelski delivered what was, formally, a plea for scientists to come to the rescue of the economy in the current socioeconomic crisis. His speech, however, can equally well be considered as a preview of the forthcoming legislation that will revise and update both the statutes of the Academy of Sciences and the research institutes belonging to the various "production" ministries.

Thus, although Jaruzelski noted that "for understandable reasons" the academy focuses its attention on basic research, his comment that "basic sciences are foundations for building practical solutions" suggests that priority in research funding may still go to those projects expected to have a major economic pay-off. Moreover, although he expressed sympathy with the scientists' anxiety over recent cuts in science spending, he noted that even in the

academy and its institutes, available resources were not always used economically and "the discipline of the research process" left much to be desired.

Jaruzelski was echoing the current concern in the Polish press that the loss of contact with Western science might cause Poland to fall irrevocably behind the leading edge of science. In such disciplines as microelectronics, bioengineering and genetic engineering, "whose development will determine our place in the world", it was essential, Jaruzelski said, not to lose contact with world science.

All these issues, Jaruzelski promised, would be included in "appropriate timetables" for implementation by the government and the academy. The scientists, for their part, will be expected to contribute to the economic recovery not only by their research work, but also Jaruzelski suggested, by providing regular briefing lectures for the party and state leadership. The academy scholars, moreover, will be expected to review their own attitudes to their work. Their task, according to Jaruzelski, is to "strengthen our place in the socialist community, to restore Poland's position in Europe and the world".

Vera Rich

UK research councils

Staffing levels blamed for waste

BRITISH research councils and the Department of Education and Science (DES) are taken to task this week for laxity in their manpower planning. An investigation by the National Audit Office, an independent body answerable directly to parliament, concludes that DES carries out no systematic scrutiny of manpower controls in the four large research councils, while the councils themselves fail to keep track of their scientific manpower needs. Unexplained increases in the proportion of higher grade posts at three research councils during the past 10 years have added £5 million to the annual civil science salary bill.

The National Audit Office, which is headed by the Comptroller and Auditor General, examined manpower controls in a wide range of public bodies that are at least partly funded by DES. The research councils, together with the British Library and British Museum, account for a high proportion of DES expenditure in such "non-departmental public bodies" and salaries soak up almost one-third of the total of £728 million spent in 1982-83. The research councils have their own staff inspection teams but do not apply standard guidelines to scientific staff.

Now the audit office questions whether the research councils have been doing the job properly. Only the Agricultural and Food Research Council and the Medical Research Council have been taking the trouble to keep DES informed of their staff in post (although it seems they could have saved themselves the time: the Comptroller and Auditor General points out that DES appears to make no use of the returns anyway). And although all the research councils have staff inspection units that are independent of line management, weaknesses of planning and coverage and poor record-keeping have made it difficult to be sure when recommendations had been followed.

Innovative scientific staff in research councils, whose work involves an exceptional degree of originality and creativity, are allowed to enjoy "fluid grading" — meaning that the work done itself depends on the occupant of a post, so that a fixed grading structure is inappropriate. The research councils have maintained that their own scientific assessments of staff gradings are adequate to keep these in line with staff gradings in the scientific civil service, and DES, hampered by a lack of staff competent to inspect scientific posts, has been inclined to leave well alone. The resulting impasse has led to anomalies.

The audit office examined staff in post at the Science and Engineering Research Council, the Natural Environment Research Council and the Agricultural and Food Research Council. There were

considerable variations in grade ratio between councils and between individual establishments, and the Comptroller appears sceptical of the explanations offered for these differences and the upward drift of gradings. The inspection of non-scientific staff by research councils is less muddled, but DES appears not to make systematic use of the information it is given.

The Comptroller and Auditor General's report will be examined by a meeting of the Public Accounts Committee of the House of Commons later this month. The

Soviet emigrants

Goldfarb's becomes test case

SIXTEEN leading Western biochemists and geneticists have appealed to the Soviet Academy of Sciences on behalf of Dr David Goldfarb, their colleague, whose exit visa for Israel was withdrawn by the KGB a few days before he was due to emigrate (see *Nature* 26 April, p.766). Dr Goldfarb is now under investigation in Moscow for allegedly trying to take with him material of importance to Soviet national security.

The KGB has confiscated Goldfarb's professional files and notes and also his collection of bacterial strains — auxotrophic mutants of *Escherichia coli* K-12 which he collected in the mid-1960s, most of which originated from the laboratory of Dr William Hayes, then at the Hammersmith Hospital, London, from Dr Yanofski's laboratory in the United States and from Dr Kunicki-Gurfinkel's in Warsaw. Only two of the strains were selected in the Soviet Union. All of them have been deposited with the two Soviet collections at the Institute of General Genetics of the Soviet Academy of Sciences, and at the Moscow Regional Research Institute of Clinical Medicine, where they are available on request to any microbiologist and, ironically, were also sent to a British laboratory in the early 1970s.

Goldfarb's work with these strains, which dealt with the use of auxotrophic strains of *Escherichia coli* as indicators of amino acids in human urine and blood plasma, is well known in the West and was even the subject of an article in the *New York Times* in 1970.

When Dr Goldfarb attempted to explain all this to the KGB, the investigating officer is believed to have replied that Goldfarb might be taking out secret strains disguised as innocuous ones. A campaign for greater "vigilance" has been instigated in Dr Goldfarb's former laboratory.

In an appeal to Dr Anatolii Aleksandrov, president of the Soviet Academy of Sciences, and to Dr Yuri Ovcinnikov, a vice-president who has several times gone on record that Goldfarb's work has no defence-related implications, Dr

committee is widely regarded as one of the most influential of Commons committees, and Members of Parliament are unlikely to hear the audit office's criticisms in silence. Most of the criticism is likely to be levelled at DES, which is supposed to take overall responsibility for overseeing manpower planning, even though individual institutions make their own detailed plans. Besides being told generally to pull its socks up, DES is instructed by the Comptroller to ensure that the fluid grading system of promotion in the research councils does not lead to a lowering of standards or the undertaking of work of higher quality than is needed.

Tim Beardsley

Goldfarb's colleagues in the West say that the harassment of Dr Goldfarb, and the seizure of his material by the secret police, is a "blatant violation of basic norms essential for the pursuit of science and international scientific cooperation. We urge you to consider the implications of similar constraints being imposed on the flow of information and materials used in basic biological research from other countries to the USSR." The appeal also asks that the Soviet Government should understand that Professor Goldfarb is being "watched with great attention by the members of the international scientific community. It is considered a test case of the willingness of the Soviet Government to comply with the principles of international scientific cooperation. Continued harassment of Dr Goldfarb will lead to grave consequences for further scientific cooperation with your country. We are prepared to take a strong public stand on behalf of Dr Goldfarb, although we hope that at this stage the problem can be resolved in a quiet way."

The signatories of the appeal are André Lwoff, Francois Jacob, François Gros, Ellie Wollman (Institut Pasteur, Paris); Sir Peter Medawar (Clinical Research Centre, UK); Sydney Brenner, Aaron Klug, Max Perutz (MRC Laboratory of Molecular Biology, Cambridge); R. R. Porter (University of Oxford), Werner Arber (Basel), Joshua Lederer (Rockefeller University), Paul Berg, Arthur Kornberg (Stanford University), Salvador Luria, David Baltimore (Massachusetts Institute of Technology) and Gunther S. Stent (University of California, Berkeley).

Vera Rich

Plant conservation — a correction

CONTRARY to the implication in the article on p.577 of *Nature* 12 April, the two databases at the Royal Botanic Gardens, Kew, are entirely separate. SEPASAT is a Kew initiative funded by Oxfam; neither IUCN nor the IUCN rare plant database receives Oxfam funds. □

US academy

Press for Soviet visit

Washington

THE US National Academy of Sciences is to re-establish links with its Soviet counterpart, having suspended relations in 1980 in protest at the exiling of Andrei Sakharov. Dr Frank Press, president of the academy, announced in his annual report last week that he would visit the Soviet Union early next month to "explore new modes of interaction" between the two scientific communities.

At a press conference following the announcement, Dr Press refused to provide details of the proposals he is taking to Moscow. But he said that the US academy had plans for new kinds of links to resurrect the relationships that existed 20 years ago, when distinguished scientists from both sides took part in exchange programmes. He said the academy's concern about Dr Sakharov was undiminished, but that its members felt it was essential to resume contact with Soviet scientists at a time of mounting international tension.

The US and Soviet academies established a formal exchange programme 25 years ago. Since then, some 500 scientists have travelled in each direction, but the volume of the exchanges dwindled after the US academy declared a moratorium over the Sakharov affair. During 1983, there was a small number of individual exchanges; but in regional meetings last year, members of the US academy called for an expansion of exchanges.

Dr Press's announcement was contained

in a wide-ranging annual report in which he excoriated Congress, the Reagan Administration and the universities for adopting policies that could hit research. He said he was concerned about the impact of "unwise and misguided" proposals within Congress to meddle with the structure of the National Institutes of Health (NIH) and the National Science Foundation, which is under pressure to place a greater formal emphasis on engineering. He criticized the administration for proposing national security controls that could stifle scientific communication. And he deplored the trend towards the use of political lobbying instead of peer review to win funds for research facilities.

These issues are expected to be high on the agenda of the Government-University Industry Research Roundtable, a new body created under the aegis of the academy to provide a new forum for discussing contentious issues in research policy. Membership of the new body, whose chairman will be Dale Corson, former president of Cornell University, was announced last week. It will include the five senior figures in federal research policy; George Keyworth, the presidential science adviser; Richard DeLauer, Under Secretary of Defense for research and engineering; Edward Knapp, director of the National Science Foundation; Alvin Trivelpiece, director of research for the Department of Energy; and James Wyngaarden, director of NIH. **Peter David**

West German education

Devolution in vogue

WEST Germany's federal government could be left without a ministry of education if an unlikely suggestion made at a meeting of the chairmen of the *Länder* (regional) Christian Democratic Union (CDU) and Christian Social Union (CSU) parties is implemented. They want to disband the Bund-Land Kommission (BLK) for educational planning and research, which is the main discussion forum where federal policies can be worked out. The suggestion reflects the strong centrifugal tendencies which have been provoked by the proposals for a new framework law for universities, and an antagonism towards the social democratic policies incorporated in the large-scale plans which have since 1973 emerged from BLK.

The chairman of the Rheinland-Pfalz CDU would like the law revoked and the entire responsibility for universities to be restored to the *Länder*. Opposition to BLK comes not only from the *Länder* with CDU administrations, but also from the remaining minority of social democratic *Länder* who are said to be unwilling to compromise with the CDU policies that BLK might now put forward.

However, partly for financial reasons, the CDU federal administration finds that small is beautiful in educational planning and it is directing BLK towards the consideration of more topical issues such as the application of new technologies and the support of gifted students. Acknowledging that some sort of consultative body is essential, the ministry does not feel threatened and expects BLK to survive the reassessment due at the end of 1985.

Another aspect of cooperation between the federal government and the *Länder* which could change is the 50:50 funding of major building projects. The excessive costs associated with the Aachen clinic (*Nature* 5 April, p.484) could be the death blow of this policy.

Problems already arise over differences between qualifications obtained in different *Länder* and consequent restrictions on university places and jobs. Greater differentiation between the *Länder* can only make these worse. Although in the long term student numbers will fall drastically, educational problems are now escalating: 1.27 million would-be students for only 750,000 university places, youth unemployment, graduate unemployment and a shortage of apprenticeships in some areas and of trained personnel in others. It looks as if one of the first jobs of the new Max-Planck-Institute for research into institutions could be to examine the educational decision-making and coordinating bodies of the federal government and the *Länder*.

Sarah Tooze

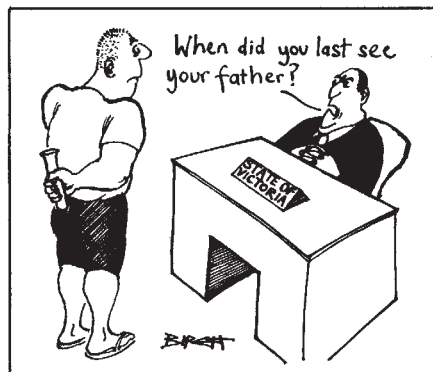
In vitro fertilization law too late

Canberra

IN Melbourne last month, legislation governing *in vitro* fertilization (IVF) procedures in the State of Victoria was deferred at least until the spring (September) parliamentary session, after opposition parties had protested that the issues needed wider discussion. The deferred Infertility (Medical Procedures) Bill (1984) was the outcome of recommendations in August 1983 by the Waller committee on the use of donor gametes in IVF. Eight of the nine committee members had supported the use of donor sperm and ova in IVF and all but two supported the use of donor embryos. Another bill, passed in the upper house and now under discussion in the lower, formalizes the status of IVF offspring as children of not, as previously, the genetic parents, but of the social parents, including those in a stable *de facto* relationship.

Many critics of the proposed legislation dispute the moral basis of the recommendations, asserting the humanity of embryos, objecting to their use for "organ bank" therapeutic purposes, freezing and thawing, experimentation and wastage and to surrogate motherhood.

With test-tube offspring now holding alumni picnics in city parks, events have overtaken the Victorian legislators, especially since the arrival in Melbourne of the world's first donor-embryo baby in



November 1983, the first IVF quadruplets in January 1984 and Zoe, the first freeze-thaw embryo baby, in March 1984. The Waller committee's report on freeze-thaw techniques and the disposition of surplus embryos is due in three months, their report on surrogate motherhood in the IVF context perhaps by December. **Jeffrey Sellar**

AIDS control

Problems of new blood test

Washington

THE announcement last month by federal officials that a blood test for acquired immune deficiency syndrome (AIDS) will be available in six months has left unanswered a number of difficult questions, both practical and ethical. The need for a huge number of tests very quickly, concern in the homosexual community about confidentiality and uncertainties over just what a positive test result will signify for someone with no AIDS symptoms may make the introduction of the test far more difficult than first thought.

AIDS researchers and blood-bank officials were concerned last week that the companies involved with Dr Robert Gallo, the National Cancer Institute investigator who isolated the AIDS virus, HTLV-III, and who has developed the prototype test, do not have the capacity to manufacture and distribute the millions of tests that will be required each year. "Our interest is that nothing stand in the way of access to the reagents" for any company ready to

produce the test, said Dr Peter Page, director of the American Red Cross Blood Services for the north-east region of the United States.

The US Public Health Service announced suddenly last week that it would accept applications — due in ten days — from companies interested in manufacturing the test. The announcement did not say whether the licence would be granted exclusively to a single company.

The two companies working with Gallo, Litton Bionetics and Biotechnology Research Laboratories, were both quickly able to produce test kits for HTLV-I after Gallo's isolation of that related virus, and expect to do as well with the virus now linked with AIDS. But according to Page, 12 million tests will be required each year just to screen the whole blood collected by the Red Cross. Several million more will be needed by the commercial blood industry, which supplies most plasma products, including factor VIII for haemophiliacs, used in the United States. Screening of high-risk groups — male homosexuals, intravenous drug users, haemophiliacs and Haitians — would take several million more tests.

Page hopes that everyone will be able to have the test at once, thus avoiding fights over which group deserves priority. There appears to be general agreement, nonetheless, that the most pressing need is for screening of the plasma products. The fact that the bulk comes from paid donors, that haemophiliacs require several treatments each month and that each treatment represents an exposure to a thousand donors, whose plasma is pooled to produce factor VIII concentrate, places the 12,000 haemophiliacs in the United States particularly at risk.

Next in priority would be whole blood collected in high-risk regions of the country, especially New York City and San Francisco. Although Red Cross officials say that their volunteer donors appear to be complying with pleas for members of high-risk groups not to give blood, several dozen cases of AIDS have been traced to whole-blood transfusions.

Abbott Laboratories has been mentioned by several researchers as a company likely to have the means to produce the HTLV-III test on a massive scale. Abbott supplies a test for hepatitis-B antigen that is currently run on every unit of blood. But mass production of the AIDS test poses special problems not shared with the hepatitis-B test. According to Cladd Stevens of the New York Blood Center, the AIDS test will use virus antigen to detect the presence of AIDS antibody in the blood, so that manufacturers must master Gallo's newly developed techniques for growing the virus.

Confidentiality, a recurring concern in the homosexual community, is sure to be

Natural selection

THE name of Stephen Gould has, since he and Niles Eldredge formulated the notion of punctuated equilibria in 1972, been dragged into the increasingly fashionable practice of arguing, mostly on specious grounds, that Darwin may have got evolution wrong. It is therefore a relief to be able to report that at the end of four hour-long lectures and a day-long seminar at Cambridge last week, Gould and eight invited discussants concluded that by and large, Darwin, who would not have known how to be specious, got it right.

Gould was in Cambridge to give the sixth annual Tanner lectures under the auspices of Clare Hall, and used the opportunity to incorporate into his already well known ideas on evolutionary processes some recent surprising insights into evolution at the molecular level. In the event, this was the only topic seriously to raise the temperature at the seminar, where eight panellists were invited to spend ten minutes each commenting on the issues raised by Gould before the discussion was thrown open to the other panellists and the floor.

In closing the meeting, Sir Peter Medawar observed with satisfaction that no theory, no matter how well established, can be considered exempt from Popperian challenge, and averred that nobody attending Gould's lectures could have left with his ideas unchanged.

Miranda Robertson

Jumping guns?

PERHAPS from a sense of neglect by Dr Robert Gallo's isolation of the causative agent of AIDS and the development of the basis for a blood test, Cellular Products Inc. of Buffalo, New York, sent out the day after Gallo's announcement a press release with the headline: "AIDS TEST AVAILABLE FROM CELLULAR PRODUCTS".

Claiming that "HTLV" had been found in 80-90 per cent of patients with AIDS or certain pre-AIDS conditions, the document went on to note that the company had been performing tests for HTLV membrane antigen since January and would, within 90 days, have a test kit available. "Response from physicians to the idea of a test kit has been very promising", the statement said. "The suspected relationship between HTLV and AIDS warrants such testing."

It took an interview with the company's president and scientific director, Dr Richard Montagna, to discover that the test is not for HTLV-III, the virus just isolated by Gallo, but HTLV-I, and that the test is not, as stated in the press release, for membrane antigen but for antibody. Montagna said the 80-90 per cent correlation they were finding was at a low dilution rate which, he acknowledged, also produced a large number of false positives.

Montagna said that "what we wanted to make people aware of is that we have been offering an HTLV test since the first of January. We certainly weren't trying to mislead anyone". He added: "We have to re-evaluate the whole thing now that we've seen Gallo's data." Stephen Budiansky

raised again when blood screening begins. Dr Roger Dodd, the National Red Cross official in charge of blood-transmissible disease programmes, thinks it likely that data gathered from AIDS testing will be handled much as hepatitis-B testing is now. A donor whose blood tested positive for AIDS would be informed by letter, an offer would be made to make the information available to the donor's physician and the donor would be entered on a confidential computer list of "deferred" donors, whose blood would not in future be accepted. Dodd said that the computer list would contain coded data indicating the reason for the deferment, but that access would be strictly controlled.

The situation may be further complicated because AIDS is a reportable disease. State health authorities are informed of donors whose blood is positive for syphilis, for example. The AIDS test may, however, escape this requirement because the test itself is not a definitive diagnosis of the disease.

Indeed, it is still unclear just what a positive test will signify. Jerome Groopman, of the Harvard Medical School, says that although studies so far suggest that everyone who has AIDS has antibody, it is far from certain that everyone with antibody will develop AIDS. "We're not going to know what to do about it except tell them not to give blood", Pages says. Stephen Budiansky

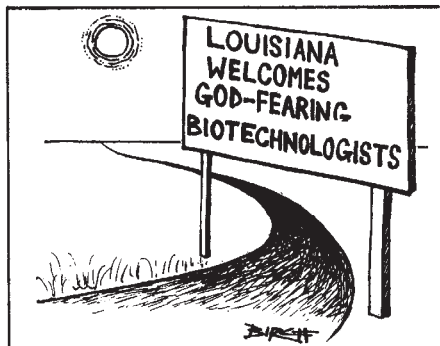
Creationism

Legal battle in Louisiana

Washington

A MAJOR effort is to begin this week in Louisiana to repeal the state law that since 1981 has required equal time for the teaching of "creation science" and evolution in the public schools. The law immediately became the target of a legal challenge from the American Civil Liberties Union (ACLU), which has involved years of legal manoeuvring largely over procedural questions. A similar law enacted in Arkansas was thrown out in 1982 by a federal judge, who ruled it an unconstitutional violation of the separation of church and state.

State legislators who are hoping to take advantage of a new climate that they believe exists in the state following last fall's elections are bringing a repeal motion



this week before the state senate's education committee. According to a head count by ACLU, the motion will be approved by the committee; its chances before the full Senate and House are less certain. Repeal forces note that the man who wrote the original bill was not reelected last year.

They also expect a sympathetic reception from the state's flamboyant new governor, Edwin Edwards. Edwards has launched an initiative to attract the biotechnology industry to the state; a proposal that he has sponsored in the legislature would provide \$25-50 million in matching funds for biotechnology research and development to companies choosing Louisiana as their base. The leaders of the repeal initiative hope to convince Edwards that the creationism law is giving this effort a black eye.

The governor is not, however, expected to come out openly in support of the repeal motion, public sentiments being what they are in conservative Louisiana. And other state officials, including the attorney general, are unabashedly enthusiastic about carrying on the fight in the courts in defence of the equal-time law. Now that the state's supreme court has ruled that the legislature can indeed dictate curriculum to the independent education board, the initial legal skirmishing is over. The way is now open for a federal court to hear the

case on its merits and to deal with ACLU's challenge to the law as a violation of the US constitution's guarantee of separation of church and state. The case is not likely to go to trial before January 1985.

Meanwhile, the state education authorities have decided not to enforce the law, which would force the state to buy new textbooks giving due attention to creationism. **Stephen Budiansky**

Biotechnology

US link for Cambridge venture

THIS week sees the formation of yet another specialist venture capital company in Britain aiming to bring developments in biotechnology to commercial success. Plant Resources Ltd (PRL), set up with £500,000 of capital and backing from various institutional investors and a specialist US venture capital fund, aims to invest not less than £10 million during the next 3 years in plant, animal and environmental technologies.

The company hopes to find a small number of projects — say five or six — that it will then support to profitability. Areas that it singles out for special interest are the use of tissue culture and recombinant DNA technology in plant breeding; the selection and development of specialized beneficial bacteria and fungi for optimizing plant growth; and the development of genetically engineered microorganisms to process organic wastes. The company is also looking at improved methods of animal embryo transfer, sex identification and the disease diagnostics and vaccines market.

The founding director of PRL, Mr Ian Kent, stresses that he will not be in competition with the larger and more established investors in biotechnology. His team will bring its business skills to projects that are at a very early stage of development. By concentrating on a few projects the company will be able to

achieve effective control of the companies it acquires or spawns.

The American connection in the new company is through Plant Resources Venture Fund, of Boston, Massachusetts, whose managing partner Mr Jack Hesse is on the board of PRL. Industry sources seem impressed by the Hesse/Kent partnership. Hesse is best known for his success in turning around the previously declining Twyford Plant Products, and Kent and co-director Robert Love come from FBC Ltd, the joint Boots-Fisons agrochemical operation that was later sold to Schering AG.

The new company is based in Cambridge, conveniently close to the new Agricultural Genetics Company, although the latter would seem to have a built-in advantage — exclusive access to the majority of developments based on research in plant biotechnology carried out by the Agricultural and Food Research Council. But Kent is not perturbed: he sees his competitor next door as a successful commercialization of one arm of civil research but feels there is no shortage of viable technology elsewhere. The problem holding back British biotechnology, says Kent, is a shortage of people able to make a link between discoveries and a viable business plan — and that is where he aims to come in.

Tim Beardsley

Nature index of biotechnology stocks

12-Month high	12-Month low	Company	Close previous month	Close 30 April	Change
14	10½	Biogen (Switzerland)	13	11¾	-2¼
2	1½	Bio-Logicals (Canada)	1¾	1¾	+¾
14¾	10⅞	Bio-Response (USA)	11	13⅞	+2⅞
14	10¼	Cetus (USA)	12⅞	12¼	+⅞
10¾	6½	Collaborative Research (USA)	8⅞	7	-1⅞
19¾	14¾	Damon (USA)	18⅞	15¾	+2¼
26¼	13	Enzo-Biochem (USA)	17½	16	-1½
10⅞	6⅞	Flow General (USA)	7¾	7	-¾
42¼	31¼	Genentech (USA)	36	35½	-½
10¾	5¾	Genetic Systems (USA)	6½	6⅞	+⅞
17¼	9½	Genex (USA)	13½	12½	-1
23	12¼	Hybritech (USA)	13½	17½	+4
16¼	11½	Molecular Genetics (USA)	14	11½	-2½
15½	10	Monoclonal Antibodies (USA)	11¼	14	+2¾
60¾	45⅞	Novo Industri A/S (Denmark)	48⅞	51¾	+2¾
22¾	16⅞	Pharmacia (Sweden)	18¾	17⅞	-¾

Closing prices are for the last Friday of the month. For over-the-counter stocks, bid price is quoted; for stocks on the American and New York exchanges, the transaction price. *Nature's* weighted index of biotechnology stocks stood at 164 on 30 April, compared with 169 a month earlier. Data from E.F. Hutton, Inc.

The lesson of Franklin Valley

SIR — I wish to dispute points raised by Professor Mulvaney (*Nature* 306, 636; 1983) and Dr Rhys Jones (*Nature* 306, 726; 1983) about my letter (*Nature* 305, 354; 1983) describing caves with archaeological contents outside the reservoir area for the Lower Gordon Scheme in Tasmania. It should not be forgotten that the search for these caves was an attempt to test the claims made by archeologists about the uniqueness of the Franklin Valley caves.

Archaeology was not taken lightly in the environmental studies, but in 1979, when these studies were carried out, the archaeologists' view was that this part of Tasmania had not been occupied¹. Officers of the Tasmanian Museum agree with this view, so that the curator's offer to accompany the Scientific Survey was not accepted. Two requests for archaeological assistance during this search were, however, unsuccessful because of the ban imposed by archaeologists on the recovery of relics.

From May 1979 until the dam was approved by parliament in June 1982, the commission was subject to a "moratorium" on further investigation (including archaeological research) imposed by the government. This ban did not apply to the Wilderness Society, the National Parks and Wildlife Service or to Dr Jones and it was during this period that they conducted selective studies within the reservoir area and the discovery of Kutikina Cave was announced.

Dr Jones' letter says that he and his colleagues have carried out systematic searches of several areas in Northern Tasmania, but he fails to mention that Southwest Tasmania has not been searched. This is a dense rainforest area that has deterred explorers, bushwalkers and archaeologists, who have deliberately refrained from investigating the Gordon Limestone in the area. Preliminary exploration was considered necessary² and carried out by methods developed by the commission for hydro-power investigation and involving the use of helicopters. Our field geologists could readily recognize karst limestone from the air and cost was negligible compared with the loss of the power scheme. Now it is up to archaeologists to follow up these discoveries, not forgetting the 300 unexplored caves in the Florentine Valley.

The main point of difference between us is that of the need to preserve the contents of the Franklin Caves in place rather than by undisturbed recovery. The Franklin Caves contain the discards of hunter-gatherers and have not been shown to contain priceless works of palaeolithic art. As they are only a few of a number of caves occupied by early man in Southwest Tasmania, the call for preservation in place is unreasonable. The commission was proposing a properly engineered undisturbed recovery for which the construction

schedule allowed a recovery period of 8 years. It would have been a fruitful period for co-operation between geomechanics scientists and archaeologists with the opportunity to develop new techniques.

I think the lesson of the whole controversy is that multidisciplinary scientific co-operation is much more fruitful than narrow confrontation.

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Indian symposium

SIR — We have read the news item in *Nature* (1 March p.6) regarding the inability of Israeli scientists to attend the VIIth International Biotechnology Symposium. To put the record straight, the organizers did everything possible to help the scientists from Israel to attend the symposium. In fact, it can be categorically stated that Indian colleagues greatly looked forward to having them at the symposium. The files of the organizing committee are open to inspection by IUPAC, the sponsor of the symposium, to confirm the large and expensive efforts put in to facilitate participation of everyone who desired and its communication with the delegates and the government to expedite the issue of entry permits/visas.

This should answer the point of your correspondent, which appears to allege that there was a deliberate attempt to exclude the Israeli scientists from the symposium. This is far from true. In fact, the Indian biotechnology community has great admiration for Israeli scientific efforts and contributions to biotechnology. We were therefore happy to see Professor Gutnick from the University of Tel-Aviv at the symposium. Normal methods of obtaining visas were available to Israeli scientists at the nearest Indian mission and our records show all missions were duly advised by the Indian authorities to issue visas to registered delegates largely as a result of the organizing committee's efforts.

Furthermore, Alitalia, the Italian airline, could have arranged for tourist visas to enable them to visit India in accordance with your correspondent's letter from Rehovot. To say as Dr Gressel has done that "the Indian Embassies concerned usually claimed to have no record of their request" to Dr Chand is clearly uncalled for.

In fairness to all, the confusion probably arose because this was the first international conference held in India after the change in the rules (withdrawal of landing

permit facility) where a large number of Israeli delegates were registered. In a democracy delay sometimes occurs and it is unfair and counter-productive to suggest that this is intentional. In fact a significant number of Israeli scientists attended meetings held before November 1982 when the practice of providing landing permits was in vogue.

T.K. GHOSE

National Coordination Committee,
VIIth International Biotechnology
Symposium,
Indian Institute of Technology,
New Delhi-110016, India

SIR — In *Nature* of 2 February (307, 408; 1984) you printed a letter from Dr J. Stefan Roken calling on scientists to boycott the International Biotechnology Symposium held in New Delhi in February, and calling for protests against the Indian Government for not permitting scientists from all countries to participate at the symposium.

The Department of Science and Technology, Government of India, has confirmed that permission was indeed given to Israeli scientists to participate in this symposium and that this fact had been communicated to Indian missions abroad. Israeli scientists did in fact participate, although not in the numbers originally expected.

P.L. SINAI

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"Wine-dark sea"

SIR — I refer to *Nature* of 12 April, p.580. Seldom can a problem — the nature of Homer's "wine-dark" sea — have resulted so rapidly in just the right research proposal to solve it. On page 12 of classified advertising, the University College of North Wales, Bangor, is seeking a Research Officer to investigate "ocean colour".

Y.M. CHAMBERLAIN

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SIR — I think Jonathan Treitel (*Nature* 12 April, p.580) is correct in ascribing this strange description to a lack of colour sense on the part of the Greeks. When I taught chemistry in Nigeria a fairly major problem was that many students never knew when indicators had changed colour; one had to hang a colour chart on the wall for comparison. This had nothing to do with eyesight, for the students in question learnt eventually, it was simply a matter of lack of practice in colour recognition. It was a little unnerving to be asked by a student clutching a piece of litmus paper "please sir, has this changed colour?". Those trained from infancy in colour recognition find it hard to believe it, but experience shows that it can be a problem.

D.A.H. TAYLOR

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Back to basics on quantum theory

Fashion seems to have come full circle as physicists look to the 1920s for inspiration. But what has prompted the change?

WHY is there, just now, so much interest in the foundations of quantum mechanics? The simplest explanation would be that the grand old men of quantum mechanics — the young men of the mid-1920s — are all now dead, so that the field is clear for a re-examination of the subject by a succeeding generation. But this is not the case. P.A.M. Dirac, who ranks with the late Wolfgang Pauli as one of those who made quantum mechanics into a coherent whole in the second half of the 1920s, is fortunately still alive and active, commuting with the seasons between Cambridge (England) and Tallahassee (Florida).

There are several candidate explanations, of which the most obvious is extrinsic to quantum mechanics as such: half a century after the ending of the most radical revision of the notion of physical reality that began with Planck (1901) and ended with the recognition that the matrix mechanics of Born, Heisenberg and Jordan (1925) and the wave mechanics of Schrödinger (1926) were different ways of saying the same sort of things, people have come naturally to a reconsideration of what really occurred during that upheaval.

The more probable explanation is practical — people's need to tell a more sophisticated generation of students a coherent tale about a field of physics which is acknowledged to be the most searching account of what the physical world is like but which has been uncomfortably balkanized almost from the beginning, and which is now not one subject but half a dozen. At one extreme there is what passes as quantum mechanics among chemists. On the convenient assumption that the nuclei of atoms in a molecule are fixed at their mean positions (for which the Born-Oppenheimer approximation provides the justification), people have become extremely skilled at calculating electronic energy levels. Another sub-branch of wave mechanics is that in which similar techniques are carried over into the treatment of the properties of nuclei. At the other extreme is that other, more esoteric, pursuit generally labelled as the theory of quantum fields from which have sprung two great successes, the successful treatment of quantum electrodynamics and the unification of the fields of electrodynamics and the weak nuclear interaction, but which seems to be running into the ground in people's attempts to construct more comprehensive unified theories (under the label Grand Unified Theory).

The obvious difficulty is that the straightforward and well-understood application of quantum mechanics to, say, the calculation of the behaviour of electrons in semiconductors cannot be presented to students as the routine calculus that it has become while the same students must know what uncertainties beset other parts of the subject.

One of the new generation of students, Joseph Godfrey from the University of Notre Dame, has now provided an interesting and stimulating pointer to the resolution of some of these questions (*Phys. Rev. Lett.* **52**, 1365; 1984). Godfrey's contribution is refreshing not merely because he thanks his fellow graduate students for "discussions and support" but also because his article is, in its way, an important historical pointer. For the starting-point is in the prehistory of quantum mechanics, the early (1927 and thereafter) contributions of Fritz London and Hermann Weyl to the argument about the meaning of the then new quantum theory. Godfrey's objective is to derive Dirac's commutator, the assumption that the operators representing conjugate dynamical variables in quantum theory anti-commute with each other in the sense that their order cannot be inverted without affecting the result, from London's paper on the subject (*Z. Phys.* **42**, 375; 1927).

Godfrey remarks that it is "unfortunate" that the importance of London's paper has been ignored all these years. That, no doubt, is true but is also understandable. London in 1927 was attempting something that must already have seemed old-fashioned to many of his contemporaries — to account for Bohr's part-classical quantization rules from dynamical principles. Eventually, London's work allowed him to contribute significantly to the understanding of superconductivity, but even in the 1920s it stimulated Weyl to generalize his earlier account of the projective geometry of the electromagnetic field to a consideration of vector fields in general and to a statement of the importance of gauge theory (as when, in electromagnetism, it is possible to multiply any solution for the vector potential of a field by complex factors without affecting the physically-observable forces).

Godfrey begins with Weyl's statement of what happens when a field vector is consistently displaced around a small circuit in relativistic space. Ordinarily, the result is to demonstrate both that Maxwell's

equations are natural and, in non-Riemannian geometry such as that of general relativity, to obtain the link between electromagnetism and general relativity in which both matter and electromagnetism contribute to Einstein's energy-momentum tensor. Godfrey's innovation is to generalize Weyl's form, the simplest, of the non-Riemannian part of the result of the displacement of a vector around a small circuit in such a way as to mix together the position of a particle and the vector-potential which it experiences. This procedure is, to say the least, interesting; Godfrey points out that it is tantamount to assuming that the geometrical properties of space are determined in part by the character of the particle being used to probe that space, "but this is what is required to derive the Dirac commutator from the formalism".

The latest in what seems to be a spate of broodings about the fundamentals of quantum mechanics is a derivation of an axiom of Dirac's from Weyl's projective geometry.

The argument (unlike London's) has the virtue of treating the position and momentum of a particle on an equal footing. The notion that the operators by which they are represented should anti-commute in the sense that the difference of their products in one order and the other should be the imaginary number $i\hbar$ (where i is the square root of -1 and $\hbar$ is Planck's constant divided by 2π) tumbles out. So too, Godfrey argues, does the exclusion principle. But by translating Weyl's geometric arguments to phase space, in which the position and momentum of a particle are treated similarly, Godfrey arrives at equations of motion for an electron even in special relativity which suggest ways in which the classical problem of the superficially infinite self-mass of a charged particle may be circumvented, as may be the corresponding renormalization procedures of Feynman and Schwinger in the case of quantum electrodynamics.

Godfrey's article is at this stage admittedly a sketch of a manuscript yet to be written (which is another of its refreshing qualities). His programme is ambitious, even for one still a graduate student. But if there turns out to be a cast-iron way of inferring the whole of quantum mechanics from some simple geometrical statements along the lines outlined by Weyl half a century ago, the consequences should be an end to the balkanization of the subject.

John Maddox

Earth science

Modelling mantle temperatures during the Archaean

from E. G. Nisbet

IN a recent paper¹ in *Geophysical Research Letters*, Jarvis and Campbell have attempted to resolve the apparent contradiction between the eruption temperatures of Archaean (older than 2.5×10^9 yr) magmas and the temperatures recorded in old continental metamorphic rocks. Thus, some komatiites (magnesium-rich Archaean lavas formed in the mantle) seem to have eruption temperatures in excess of $1,650^\circ\text{C}$ (ref. 2), whereas some metamorphic assemblages³⁻⁵ preserved in granulitic rocks formed deep in Archaean continents seem to imply that, in some places at least, the heat flux into the base of the continents was approximately the same as it is today, and that the mantle temperature was not much hotter than the current $1,350$ – $1,400^\circ\text{C}$.

Jarvis and Campbell have constructed a numerical model of whole-mantle convection and found that mantle temperatures at least 200°C more than exist at present lead to a highly disordered form of convection in which the horizontal thermal boundary layers are thinned to about 30 km and become highly unstable. Random portions of the cold surface layer would break off and fall into the interior and the continental crust could not survive. As there is good field evidence that large stable areas of continents have existed since early Archaean time, the implication of the Jarvis and Campbell model is that the Archaean mantle must have been cool enough — possibly only 100°C hotter than today — to avoid such disorder.

How, then, can the high eruption temperatures of the komatiites be accounted for? Jarvis and Campbell appeal to the only possible source in a whole-mantle convection model — the lower thermal boundary layer at the core-mantle boundary.

They suggest that komatiites were derived from hot plumes rising from instabilities of this layer, the hot parcels of mantle beginning to melt at about 450 km below the surface as they cross the solidus, and then reaching high degrees of partial melt before eruption. They go on to explain the 'cool' metamorphic evidence by suggesting that the recorded temperature profiles come from rocks formed near Archaean subduction zones where geothermal gradients were low. Thus their model manages to accommodate the metamorphic data but at the price of banishing the komatiites to the infernal regions.

The model is interesting and may well have much truth in it, but is based on some questionable assumptions. One is that viscosity was spatially uniform but ex-

ponentially dependent on the mean temperature of the convection layer. No attempt is made to investigate the effects of possible chemical stratification in the mantle⁶ on the viscosity and solidus: if a refractory dunitic uppermost mantle existed, or if a magma ocean lay in the lower part of the upper mantle, the variation in viscosity would be sharply discontinuous. Furthermore, since komatiites are abundant in the Archaean geological record and may have been the parent liquid to mid-ocean ridge lavas, the required number of plumes rising from the core-mantle boundary may be unreasonably large.

England and Bickle³ have recently tackled the problem by investigating the 'cool' metamorphic gradients and then exploring their consequences; they point out that the relationship between the eruptive temperatures of komatiites and the temperature of the mantle is poorly understood. If the continents were indeed cool, then the heat flux of the surface of the Earth was probably out of equilibrium with the generation of internal heat (which was three to four times greater then than now). They point out that if temperatures at the base of the continental crust were more than a couple of hundred degrees higher than they are now, the Archaean lithosphere would not have been able to support the mountain heights that are implied by the metamorphic pressure data. They also conclude that the forces driving orogenesis in the Archaean were comparable in magnitude to modern forces — around $5 \times 10^{12} \text{ N m}^{-1}$.

Both these models favour mantle temperatures close to modern values. What of the 'hot' point of view? A different starting point is to assume, simplistically, that the komatiites really do reflect typical mantle conditions and to investigate the consequences. What if the mid-ocean ridges were fed by a komatiite parent liquid^{7,8}? A komatiite oceanic crust overlying a hot olivine-rich lithosphere and upper asthenosphere would be much hotter at comparable depth than modern oceanic plate, and thinner on subduction. Compared with present conditions, such a system of plate tectonics might allow a greater proportion of the total surface heat flux to be carried by the creation and destruction of plates, the plates being thinner, hotter and faster-moving.

Extrapolation of the available information about the densities of olivine and of magnesium liquids suggests that, at depth, olivine would float in komatiite liquid⁶. If so, a magma zone may have existed at depth. Such chemical

stratification would have a major control on heat transfer within the mantle and it is possible that to some extent the heat flux depended on the mechanisms controlling ascent of magma from this molten zone. Perhaps the oceanic plates dissipated much heat but the continents were relatively cool. Much depends on the continental lithosphere which may have been less thick than today but perhaps more refractory. The distribution of radioactive elements within the crust and mantle may also have been different.

All this modelling depends on field evidence which is complex and difficult to interpret. The maximum MgO content (and hence eruptive temperature) of komatiite liquids is not well known although 32 per cent ($1,650^\circ\text{C}$ or more) is commonly assumed. Metamorphic 'geotherms' do not represent thermal equilibrium of an isochronous column of rock, even if they are derived from rock assemblages which somehow managed to approach thermodynamic equilibrium. For this reason, England and Bickle's study of the controls on pressure, not temperature, is most interesting. At what stage would continents overthickened by deformation simply spread out?

Isostasy comes in here. Erosion also controls continental thickness, which has probably always been much as today⁹. But was there water in the ocean and what was its level? Were the mid-ocean ridges so high that they protruded, or even tipped out the water onto the continents? And were the Archaean oceans deeper?

The depths of the oceans and the heat flux problem have wide significance. It is possible that some of the first progenotes inhabited early Archaean hydrothermal systems in the same way that archaeobacteria occupy black smokers on modern mid-ocean ridges. Thus the nature of the ridges — their chemical composition and temperature, the water depth, the amount of heat dissipated in hydrothermal systems close to the ridge and hence the extent of interaction between new oceanic crust and seawater — are all of major interest in considering the origin of the biosphere and the controls on its evolution in time. Indeed, by stabilizing temperatures and allowing for water in the oceans, life may have exerted a profound control on the Earth's tectonic regime. □

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Cancer biology

Recessive mutation in aetiology of Wilms' tumour

from Ellen Solomon

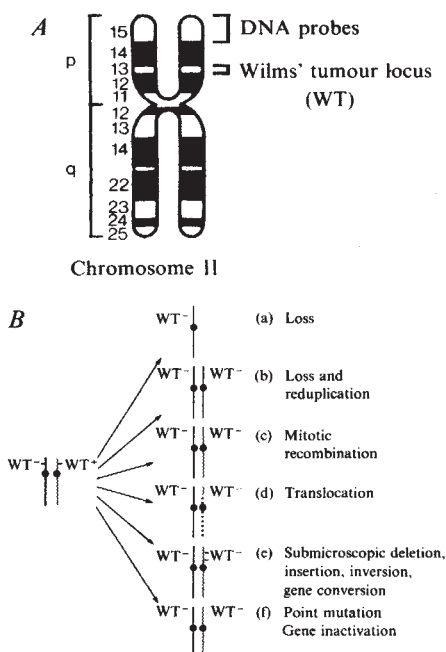
MUCH has been written and published in recent months about the cellular genes whose activation by retroviruses, mutation and chromosomal rearrangements seems to convert them into dominantly acting oncogenes involved in tumour initiation or promotion^{1,2}. Less has been heard of tumours which seem to be the result of the deletion or inactivation of both copies of a wild-type genetic locus and therefore to be the result of a recessive mutation. The only specific recessively acting mutation to have been studied in any detail until now is that associated with rearrangements of chromosome 13 in the childhood tumour, retinoblastoma^{3,4}. But pages 170 to 178 of this issue of *Nature* contain reports from four independent laboratories of genetic changes in Wilms' tumour resembling those documented in retinoblastoma⁵⁻⁸.

Like retinoblastoma, Wilms' tumour occurs in childhood in both inherited and spontaneous (sporadic) forms. In a small percentage of both forms, a specific deletion of band p13 of chromosome 11 has been observed. This deletion provides a clue as to which chromosomal region might be involved in Wilms' tumour and, along with the hypothesis of Knudsen that at least two mutational events are necessary for tumor formation⁹, provides a basis for the investigations. Thus if a gene on one of the pair of chromosome 11s is deleted or inactivated, a second mutational event on the homologous chromosome is postulated to be necessary for tumour formation. Since the nature and precise location of the inactivated gene is unknown in both retinoblastoma and Wilms' tumour, small structural changes cannot be looked for, and it has therefore been necessary to focus on evidence of gross chromosomal changes within the tumours which might result in the deletion or rearrangement of the second locus. All four papers in this issue show that in a large proportion of Wilms' tumours, such changes can be seen.

Several different mechanisms, shown schematically in the figure, could account for inactivation or deletion of the homologous locus. Among the events involving whole chromosomes that can be detected without knowing which locus to examine are (a) loss of a whole chromosome so that only one homologue remains (hemizyosity), (b) loss of one chromosome and reduplication of the other (homozygosity), (c) mitotic recombination (crossing-over between chromosome homologues in somatic cells during mitosis) so that the part of the chromosome containing the mutation becomes homozygous and (d) translocations leading to

deletion or inactivation of the locus.

The methods used to investigate mechanisms a, b and c make use of the naturally occurring genetic variations found between chromosome 11 homologues. These are detected with DNA probes that identify differences in sites of cutting of various restriction enzymes (restriction fragment length polymorphisms). In normal tissue, a certain number of sites on the homologues



A, normal chromosome 11 showing position of the deletion (11p13) seen in patients with Wilms' tumour and the area covered by the DNA probes. B, mechanisms for generating mutation at normal Wilms' tumour locus (WT+) in a cell already containing one inactive or deleted allele (WT-).

will differ, the chromosomes being heterozygous at these sites. The new papers report a comparison between normal tissue and tumour tissue of Wilms' tumour patients with respect to such heterozygosity. The DNA is cut with appropriate restriction enzymes and Southern blot analysis performed with a variety of probes, all directed to the tip of the short arm of chromosome 11. The probes recognize sequences flanking the insulin gene, β -globin gene, parathyroid hormone gene, the cellular homologue of the transforming gene of Harvey murine sarcoma virus (c-Ha-ras1) and a random human genomic sequence. The salient observation from all the studies is that in cases where normal tissue is heterozygous, the tumour is often homozygous — the predicted result of loss of one chromosome or recombinational events as in mechanisms a, b and c. The end

result is loss of the functional 'Wilms' locus' from both chromosomes.

While in the retinoblastoma studies it was possible to distinguish mechanisms a and b from c, and to demonstrate examples of each, this was not possible in any of the studies on Wilms' tumour. The problem is largely technical, caused by lack of DNA probes for any region of chromosome 11 other than the tip of the short arm. To detect mitotic recombination in Wilms' tumours, further probes, particularly for the region between 11p13 and the centromere, will be needed. Whatever mechanism causes it, homozygosity is present in four of six tumours investigated by Fearon *et al.*⁸, in five of seven by Koufos *et al.*⁵, in two out of two by Reeve *et al.* (including one case of hemizyosity)⁷, but in only one of seven by Orkin *et al.*⁶ (probably by chance rather than because of anything unusual about the particular patients or methods). Combining these results, gross chromosome changes seem to result in homozygosity for all or part of chromosome 11 in 55 per cent of Wilms' tumour patients, a very similar figure to that recorded for retinoblastoma. Presumably the other 45 per cent of Wilms' tumour patients have more specific and smaller regional mutations, such as produced by mechanisms e and f in the figure.

Although the studies of Wilms' tumour have failed to produce evidence of mitotic recombination, as was so elegantly shown for retinoblastoma, they do provide an example of chromosomal translocation, mechanism d. Reeve *et al.* find that one of their tumours contains a translocation between chromosomes 11 and 12 which has resulted in the loss of an allele of c-Ha-ras1. Although no loss of material could be detected cytologically, there has presumably been a submicroscopic deletion in chromosome 11. The details are somewhat perplexing because c-Ha-ras1 is now believed to be sufficiently far from 11p13 that if the deletion included the Wilms' locus, it would almost certainly be visible¹⁰. Therefore, it seems more likely that the chromosome translocation which resulted in c-Ha-ras1 deletion caused inactivation of the Wilms' locus than its deletion.

The major conclusions from this work are, first, that somatic mutational events are at least as likely to be caused by gross chromosomal changes as by more site-specific events; and second, that recessive mutations may be an important contributing factor to oncogenesis. The fact that both retinoblastoma and Wilms' tumour are embryonic tumours, with a significant genetic component, may or may not be significant. Other categories of tumours need to be studied in a similar fashion. Obvious candidates are other childhood tumours, such as neuroblastoma; inherited tumours of adults, such as medullary thyroid carcinoma and familial polyposis coli; and sporadic tumours with later age of onset, such as colonic adeno-

carcinomas. In none of these cases is it obvious which chromosomes to examine. Chromosome preparations from solid tumours are notoriously difficult to analyse and in few cases other than retinoblastoma and Wilms' tumour has a very specific alteration been seen. At present, therefore, either clues must come from the inconsistent, but perhaps significant, chromosome changes in these tumours, or enough probes must be used to cover all the chromosomes. Once the location of any recessive mutations is found, attempts to identify the normal gene products by molecular genetic analysis will follow. In the case of inherited tumours,

genetic counselling will be greatly helped by the ability to determine which parental chromosome carries the mutant allele. □

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Ecology

Different ways of being a mouse

from John H. Lawton

WHY are some assemblages of animals and plants richer in species than others? Answers to this question take various guises, but usually hinge on G.F. Gause's venerable 'competition exclusion principle'. In Gause's own words, "as a result of competition two similar species scarcely ever occupy similar niches, but displace each other in such a manner that each takes possession of certain peculiar kind of foods and modes of life in which it has an advantage over its competitor"¹. The possibility that major differences in species richness can be solely accounted for by niche differences between species has tended to be played down, particularly for plant communities. After all, for growth every plant requires water, light, space and three or four key limiting nutrients — not leaving much scope for niche preference. In fact, this commonsense view is almost certainly wrong, as David Tilman has recently pointed out for plants²; on page 150 of this issue of *Nature*, Abramsky and Rosenzweig make a similar claim for desert rodents³.

For Gause to be right, there must be 'more ways of being different' in species-rich than in species-poor assemblages. Simple mathematical models of competing species show, however, that as the number of competitors, n , increases, $2(n-1)$ inequalities must be satisfied before stable coexistence is possible⁴. In biological terms this means that as n increases, it gets harder and harder to find sufficient differences between species to maintain coexistence. Three theoretical possibilities bypass the constraints of this simple model and have the potential to explain species-rich systems without competitive exclusion. First, competition for limiting resources might be prevented by the impact of predation⁵⁻⁷. Second, if, instead of living in a spatially uniform world, as simple models assume, potential competitors have independently aggregated distributions, competitive exclusion becomes extremely

unlikely⁸. Third, if real populations are not in the stable equilibrium frequently assumed by modellers, competitive exclusion can be prevented by repeated environmental disturbances⁹.

Perhaps because of their relative novelty, these and related ideas tended to hog the limelight in recent years; there did not, before Tilman, seem to be much new to say about Gause's 'ways of being different'. Tilman uses graphical, rather than mathematical, models to explore coexistence mechanisms in sets of species, each differing in its sensitivity to the ratio of two limiting resources. Contrary to received wisdom, his results demonstrate that niche diversification and coexistence are theoretically possible for species-rich assemblages competing for just two essential limiting resources. Moreover, the models lead to some very clear predictions. For example, they suggest that a graph of species richness against total supply of resources should be humped, rising rapidly to a peak in relatively resource-poor habitats, and thereafter slowly declining with increasing supply of resources.

Tilman's arguments mainly concern plants competing for nutrients — nitrates and phosphates, for example. Abramsky and Rosenzweig³ now find that the number of coexisting rodent species in two different desert habitats, one rocky and the other sandy, first rises quickly, then falls more slowly along a gradient of increasing rainfall, exactly as predicted by Tilman.

As with all interesting observations, the data generate more questions than answers. First, if Tilman's explanation holds, what are the two types of resources partitioned by the mice? They are unlikely to be anything as simple as concentrations of nitrates and phosphates. Second, an important assumption underpinning Tilman's predictions is that the two resources cannot be substituted — a deficiency of a cannot be made good by using more of b — and that they are independently and patchily

distributed across the habitat. Basically, this permits each plant species to specialize on a different ratio of the two resources, and for each species to take those special requirements from different microhabitats in a spatially heterogeneous world. As Tilman points out, the mobility of animals, combined with a tendency for their resources to be able to be nutritionally substituted — a deficiency of a can be made up by eating more of b — is likely to undermine both his assumptions and his predictions. In sum, if mice use resources that can be substituted and/or forage in many different places, Abramsky and Rosenzweig's species-density changes cannot be due to the mechanisms postulated by Tilman. But if the resources used by the mice can be only partially substituted, and if each species is a microhabitat specialist, as seems possible³, then something akin to Tilman's model may well be operating in this system.

Of course, the data could also be explained by entirely different mechanisms. A simple possibility is that rodent species-richness is determined by plant species-richness, with only the plants behaving as Tilman suggested. An alternative possibility, discussed by Abramsky and Rosenzweig, is that the patterns are driven by environmental disturbances (negating another of Tilman's crucial assumptions, namely that populations exist close to equilibrium). It should be noted that Connell's hypothesis⁹ also predicts that species-densities will first rise and then fall along a gradient of decreasing environmental disturbance.

Obviously, more needs to be known both about the mice and about their habitats. The results of further research are likely to be particularly interesting in two ways. First, the relative importance of equilibrium and non-equilibrium dynamics in community ecology is far from clear^{10,11}. Second, the importance of habitat heterogeneity in determining the outcome of interspecific competition in the field deserves more attention^{2,8,12}. Hence a clearer understanding of the mechanisms underpinning the patterns documented by Abramsky and Rosenzweig could have implications for community ecology extending well beyond the somewhat esoteric question of how many different ways there are of being a mouse. □

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Retinal physiology

A foot in the vitreous fluid

from A. R. Gardner-Medwin

A PAPER by Newman on page 155 of this issue documents a striking property of the glial cells in the vertebrate retina: their membrane permeability, which is almost exclusively a permeability to potassium, is largely concentrated at the end which has membrane expansions ('endfeet') facing the vitreous fluid of the eye¹. Indirect arguments in support of the idea of regional specialization of the glial membranes had already been put forward by Tomita and his colleagues^{2,3} and by Newman himself^{4,5} on the basis of experiments on the frog retina. Newman's new experiments provide a particularly direct demonstration of the phenomenon for glial cells isolated from the salamander retina. The elegance of the experiments may help to stimulate a reconsideration of the role of the glial cells in the vertebrate retina.

Like glial cells throughout the nervous system, Müller cells — the principal glial cells of the retina — do not, so far as is known, participate directly in the processing of neural information. As the only cells that span the full depth of the retina (see the figure), Müller cells are in a position to influence, or to be influenced by, the environment of all the neurone and receptor types of the retina as well as the vitreous fluid.

The principal issue for debate in Müller cell electrophysiology has been the part the cells play in the generation of the electroretinogram — that is, the changes in potential generated across the retina and the eye in response to light. Miller and Dowling⁶ suggested that the vitreal positive 'b' wave of the electroretinogram might be due to a build-up of extracellular potassium in the retina following neural activity and to the consequential currents flowing through the Müller cell. That suggestion seemed unlikely to be correct when measurements with ion-selective electrodes showed that potassium is largely built up close to the vitreal surface of the retina⁷⁻⁹, on the assumption that the permeability of the Müller cell is uniformly distributed, this would lead one to expect voltage changes opposite in sign to those observed for the b wave. But with evidence of the asymmetry of the permeability of Müller cells¹⁻⁵ it becomes possible to account for the observed polarity of the b wave even for contributions coming from increases in potassium concentration close to the vitreal surface. In fact, the greatest contribution to the electroretinogram will come from the changes in potassium concentration that occur furthest from the vitreal surface, since for these the current loop through the Müller cells passes through the greatest extracellular resistance. The distal increase of potassium

concentration is more transient than the proximal increase, partly because of uptake of potassium into the photoreceptors. This seems adequately to explain why the b wave is substantially briefer than the depolarization measured within Müller cells^{4,5}. Thus the potassium hypothesis for generation of the b wave looks as if it may be substantially correct.

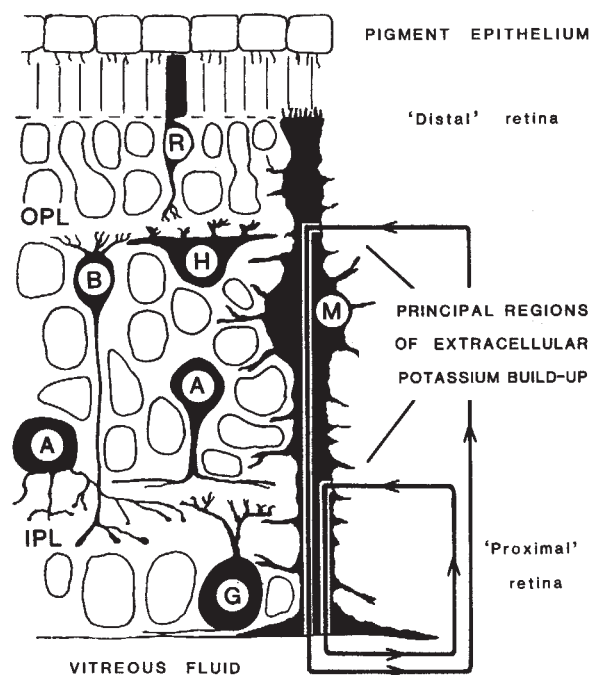
A more important issue than the generation of the electroretinogram is the possible effect that Müller cells may have on retinal neurones. In other tissues it has become clear that glial cells have a significant role in dispersing potassium from regions of the neural environment where it has built up (see review in ref. 10). This is the so-called 'spatial buffer' action. Newman suggests that in the retina, the specialized endfeet of Müller cells serve to transfer potassium not to other regions of nervous tissue but specifically to and from the large reservoir of vitreous fluid. Since the retina is only 0.2 mm thick and often subject to uniform stimulation, there may be little undisturbed neural tissue following retinal stimulation and the fluid bordering the retina may represent the chief reservoir of extracellular fluid available for buffering. Newman's proposal is therefore attractive. It remains to be established, though, whether the effect on the neuronal environment is sub-

stantial. Using data from Fig. 7 of Newman's current-density analysis^{4,5} it is possible to calculate that the cumulative effect of current flowing in the loops shown in the figure during the first 2 seconds of the b wave would diminish the extracellular potassium concentration by approximately 0.15 mM, on average, throughout the retina. The decrease would be two to three times greater (0.3–0.5 mM) in the localized regions of potassium build-up where the current enters the Müller cells. These changes, if confirmed experimentally, seem likely to be large enough to have a significant effect on retinal neurones.

If direct and rapid clearance of potassium through the Müller cells into the vitreal fluid turns out to be important for the homeostasis of the retinal environment, it should stimulate consideration of similar mechanisms in other tissues. It may also stimulate a reconsideration of some data obtained from retinal preparations in which the vitreal fluid has been removed. □

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The principal cell types and the principal paths of current flow due to potassium build-up following light stimulation in the vertebrate retina. The current is carried largely by potassium within and across the membranes of the Müller cells and largely by sodium and chloride in the extracellular space: it serves to transfer potassium from the extracellular space to the vitreous fluid. A, amacrine; B, bipolar; G, ganglion; H, horizontal; M, Müller cells; R, receptor cells; IPL and OPL, inner and outer plexiform layers. (After refs 4,5 and 11.)

Evolution

Evolutionary case histories from the fossil record

from Adrian Lister

THE revitalized debate about rates and modes of evolution signalled by Eldredge and Gould's 1972 paper, 'Punctuated equilibria: an alternative to phyletic gradualism' (in *Models in Paleobiology*, ed. Shopf, T.J.M., p.82, Freeman, San Francisco), has focused on several rather distinct propositions which are not necessarily all true or false together, and should be tested separately. First, does the evolution of form in a lineage proceed in a slow, fairly regular manner over long periods of time, or does it occur in bursts of short duration interspersed with much longer periods of little or no change? Second, does most evolutionary change occur in association with speciation events (the splitting of a lineage to form two species)? Third, is major evolutionary innovation commonly associated with small population size? And fourth, if change does occur in a rapid burst, is it through a chain of intermediates ('fast gradualism'), or are there actually jumps in morphology ('saltations'), without intermediates? There has been extended theoretical discussion of the genetic and ecological plausibility of these alternatives; modern genetics would seem to offer suitable mechanisms for various different modes. The fossil record, despite its problems and imperfections, is uniquely placed to furnish examples of what actually happened, as was apparent at a recent symposium*.

Three main patterns might be observed in a chosen lineage: stasis (little or no change), gradual change, and punctuation (very rapid or sudden change). In addition, the putative ancestor may either persist alongside its descendant, implying a split into two species, or may not persist, implying transformation of the entire ancestral species. Contributors at Swansea were acutely aware of the various biases whereby an observed pattern of change in a fossil sequence might give a misleading impression of the true evolutionary rate or mode. In particular, for an observed 'punctuation' to be direct evidence of rapid evolution, it must be explicitly demonstrated to be not merely the result of a break in deposition, nor the sudden immigration of a form which had evolved (at an unknown rate) elsewhere, but a genuine *in situ* transformation. This requires sampling of finely stratified correlated geological sequences over a wide geographical area.

Most of the presentations concerned marine invertebrates, which demonstrate a wide range of evolutionary patterns. Very

extensive sampling of Cenozoic ostracod lineages (Crustacea) from the south-west Pacific reveals a 'punctuated equilibrium' pattern of evolution in association with sea deepening (R. Whatley, University College of Wales, Aberystwyth). *Poseidonamicus rudis* is one of many species which display stasis in shell ornament over millions of years, but which during the Cenozoic rapidly gave rise, in allopatric (geographically distinct) populations, to species with different shell ornaments, which themselves then display stasis in shell ornament. (Over the same period, shell size in these lineages slowly increased by gradualistic evolution.) Similarly, among Mesozoic brachiopod taxa, many species display long-term stasis worldwide (D. Ager, University College of Swansea), and there is one example of the origin of a new form by allopatric speciation: in the Lower Jurassic, *Homoeorhynchia meridionalis* arose from a marginal population of *Homoeorhynchia acuta* (Ager, D.V. *Palaeontology* 26, 555; 1983). By contrast, geographical sampling of the European Jurassic scallop *Radulopecten fibrosus* suggests the rapid appearance of a new shell form in the sole contracted remnant of the species, rather than in an allopatric part of it (A. Johnson, University of Leicester). Again the transition punctuates long periods of stasis and is believed to have taken no more than a few tens of thousands of years.

Varied modes of evolution were documented among the ammonites. Late Cretaceous lineages studied by W. Kennedy and C. Wright (University of Oxford) show a pattern of 'punctuated gradualism', with the sudden appearance of altered shell forms, as well as slow gradualistic changes in size, ornament and sutural complexity. In Lower Jurassic Lipoceratidae, geologically instantaneous events produced *Androgynoceras centaurus* from a *Lipoceras* stock with survival of the ancestor (that is, by speciation), and subsequently *Amaltheus stokesi*, apparently without survival of the ancestor. Between the punctuation events are periods both of stasis and of slow gradualistic change in size and form, but the punctuation events are regarded as the more innovative of the modes (M. Phelps, University of Lyon). Similarly, studies of various trilobite and graptolite genera suggest that both punctuated and slow gradualistic evolution occurred, but that the latter accounts for no more than 10 per cent of observed phylogenetic change (R. Fortey, British Museum).

Particularly convincing examples of slow gradualistic evolution are provided

by lineages of planktonic Foraminifera (Protozoa), which change stepwise through many horizons, the same sequence being found at sampling points across the geographical range (F. Banner and F. Lowry, University College London). Thus in *Globorotalia fohsi*, the top of the shell became progressively more pointed through 1.6 Myr of the Miocene. Among the Mammalia, both the Eocene primate *Microchoerus cf. erinaceus* (M. Godinot, University of Montpellier) and the Pleistocene vole lineage *Mimomys-Arvicola* (A. Stuart and K. Joysey, University of Cambridge), sampled in the West European part of their range, change in body size and dental morphology via intermediate stages, implying gradualistic evolution.

There was a strong air of pluralism at the conference, with many participants allowing roles for both rapid and slow cumulative change in the history of life, as well as the common occurrence of stasis. However, one should resist the temptation at this stage to 'head count' the examples of each mode for an estimate of their relative importance. Slow gradual evolution can be seen to achieve moderate change of size and form, but whether major phyletic transformations are produced by an accumulation of such transitions is difficult to establish because we do not have long enough series of fossils. Equally, our ability to recognize punctuated change falls off the greater the transition which has occurred, not least because we may simply not realize that the ancestor and its descendant are related; and many examples of 'punctuation' are still lacking the geographical control to distinguish rapid evolution from rapid immigration. Finally, our sample of case histories is inevitably biased by the attention given to examples which provide a clear story.

The issue of whether slow gradualism and punctuated equilibrium are differentially associated with particular animal groups or ecological situations will increasingly be discussed. Do communities with complexly interlocking adaptations tend to stasis, interrupted by occasional rapid episodes when many species change together? Are simpler communities with broader niches more tolerant of gradual shifts in adaptation? To what extent are stasis, or evolution and its rate, determined by the stability or rate of change of the environment? At Swansea, it was suggested that among trilobites and graptolites, punctuated equilibrium predominated in the more complex benthonic habitat, while phyletic gradualism was commonest among the pelagic and planktonic forms in simpler grazing niches (Fortey). In Pacific benthonic ostracods, the pattern of stasis and rapid speciation seen in deep-sea forms contrasts with the common occurrence of slow gradual change in shallower-water species (Whatley). Examples of evolution driven by environmental change were more common than examples of evolution in an apparently unchanging environment.

* The symposium 'Evolutionary case histories from the fossil record' was held at the Annual Conference of the Palaeontological Association, University College, Swansea, 17-20 December 1983, and organized by J.C.W. Cope and P. Skelton.

Adaptive evolution of shell shape in the Carboniferous bivalve *Carbonicola* can be directly related to changes in sedimentary conditions (R. Eagar, Manchester Museum). Transformations in Mesozoic brachiopods were in most, though not all, cases associated with environmental changes such as sea deepening (Ager).

The important question of whether rapid evolutionary change occurs by genuine saltation or by 'fast gradualism' is in most cases beyond the resolution of the fossil record, as is the question of the pattern of genetic change underlying observed morphological transitions in general. Information of genetic significance may nonetheless be present in some palaeontological data sets. For instance, in both Johnson's scallop *Radulopecten* and Phelps's ammonite *Oistoceras*, variants resembling the descendent form are present in the latest ancestral samples before the punctuation event. In other examples the nature of the developmental switch can be inferred; thus in some ammonite lineages, failure of growth of the outer

whorls left the inner (youngest) whorls to form the adult morphology of the descendent (Kennedy and Wright). The potential for unusually finely stratified fossil sequences capable of resolving evolutionary modes may be greatest in the Pleistocene, the most recent geological period. Thus at one locality, the vole, *Microtus oeconomus*, has been sampled across six levels within the 10–15,000 years of a single interglacial, and stepwise size change documented (Stuart and Joysey).

The complexity of the evolutionary process and the problems associated with fossil evidence make it all the more pressing that palaeontologists collect their samples extensively and with precision, and keep the various possible explanations for a given set of data and ways of deciding between them clearly in mind. The potential rewards remain great: fossil sequences which pin down evolutionary modes, while rare, are within our grasp. □

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Earth science

What triggers reversals of the Earth's magnetic field?

from J. A. Jacobs

It has long been tempting to suppose that there is a correlation between changes in the Earth's magnetic field, whose origin lies in the Earth's core, and events at the Earth's surface, but there has been little convincing evidence of such a correlation (see refs 1 and 2 for a sceptical view). Although changes in the magnetic field do sometimes coincide with changes in global ice volume, short-period rapid glaciations and climatic cooling, there is no proof that the relationship is one of effect and cause; indeed magnetic excursions have also occurred at times when the oxygen-isotope curves from deep-sea cores do not record major changes in ice volume or rapid cooling³. So is it possible to relate reversals of the Earth's magnetic field to other events, given the problem of establishing any correlation for lack of an accurate chronology?

Olson⁴ has recently investigated the behaviour of the Earth's main magnetic field during a polarity transition using the α^2 dynamo model in a turbulent core. He shows that reversals of the dipole field occur with changes in sign of the helicity (a measure of the correlation between turbulent velocity and vorticity). Interestingly, long-term fluctuations in the helicity lead to a reversal of the dipole field, but not the toroidal field, whereas both poloidal and toroidal fields can reverse following short-term fluctuations. Olson explains the reversals in terms of fluctuations in the level of turbulence in the Earth's core caused by two competing energy sources —

heat loss at the mantle-core boundary and progressive growth of the inner core. These produce helicity of opposite sign, the α -effect generated by growth of the inner core tending to oppose and destabilize the α -effect generated by heat loss at the mantle-core boundary. One possible shortcoming of Olson's analysis is his neglect of the Earth's non-dipole field, which plays a key part in many other theories.

The presence of two energy sources generating opposite helicity at the two boundaries of the outer core provides an attractive explanation for the triggering of reversals in polarity. The equations of motion governing conditions in the outer core permit a magnetic field of either sign; changing boundary conditions seem to be the most likely cause of a change in polarity. Prior to Olson, Schloessin and Jacobs⁵ had suggested such a model in which changing conditions at the mantle-core boundary are caused by the addition of lighter material from the outer core which rises as heavier material precipitates out to form the inner core⁵. In their model, the relative magnitude of the growth rates at the mantle-core boundary and at the inner core boundary determines the polarity of the field. Stevenson⁶ has also suggested the existence of two energy sources in the Earth's outer core — but not as a mechanism for causing reversals. He suggested the geodynamo was powered by thermal convection during the early history of the Earth but that this power source died about

10⁹ years ago to be replaced by the energy released by the growth of the inner core.

Gubbins has recently considered the possibility that reversals are initiated by such external phenomena as tectonic events, ice ages and the fall of tektites⁷. Investigating the change that such events would have on the pressure in the core — which would in turn affect the rate of freezing of the liquid outer core and modify the power supplied to the dynamo — he estimates that a pressure change of only 1 bar over 10,000 years could supply the power requirements of the dynamo. Even so, none of the external effects that he considered seems likely to be able to cause even so small a change in pressure although such an explanation cannot be ruled out because of uncertainties in the values of the parameters, the time scale for transmission of a pressure change through the mantle and the magnitude of the increment in the power supply necessary to cause reversal.

More recently, Force⁸ has suggested a relationship between geomagnetic reversals, seafloor spreading rate, palaeoclimates and black shales. Clearly he is really only presenting a broad correlation; when it comes to individual reversals Force has to admit that "many of the linkages proposed are matters of dispute or incomplete work". His only suggestion of any connection with the Earth's magnetic field is a reference to Sheridan *et al.*⁹ who end their paper with the observation that fast rates of seafloor spreading occurred during both the Jurassic and the Cretaceous quiet (magnetic) zones, "suggesting that plume eruptions from the lowermost mantle might connect these two processes and explain the pulses of fast spreading as well as the decrease in reversals of the magnetic field". It is difficult to visualize the physics of such a process; erupting plumes would be more likely to cause increased magnetic disturbances (more frequent reversals) than create magnetic quiet conditions.

It is clear that until we have a better understanding of the mechanism of generation of the geomagnetic field and of the morphology of the transition field, any correlation of reversals with surface phenomena must remain highly speculative. Whether a common source (as suggested by Sheridan *et al.*⁹) can initiate major changes in both the magnetic field and surface phenomena, or whether changing conditions in the core can influence surface phenomena, or vice versa, remains to be seen. □

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Darwin's gradualism and empiricism

SIR — In his recent Commentary, Rhodes¹ details how Darwin "specifically repudiated" three of the four tenets attributed to Darwinian gradualism by Eldredge and Gould², architects of the hypothesis of evolution by "punctuated equilibrium". The fourth tenet is "shared in substance by all who accept the fact of evolution". Rhodes further shows that Darwin subscribed to "both of the two important consequences of punctuated equilibrium."

It is surprising, then, that Rhodes concludes: "The hypothesis of punctuated equilibrium is of major importance for palaeontological theory and practice. Several significant tenets of this view were enunciated by Darwin... [but] it would be misleading to imply that he anticipated every nuance of the theory."

Which nuances were unanticipated by Darwin? How is saltational "punctuated equilibrium" different from Darwin's gradualism? Eldredge and Gould² represent (misrepresent) gradualism as the equivalent of orthogenesis³. Now Rhodes has shown that Darwin's gradualism is very similar to "punctuated equilibrium" in outward appearance. However, gradualism and any form of saltation differ fundamentally in theory, and this difference deserves discussion.

A gradual process advances by steps, and intermediates are present as evidence of transition. Darwin's gradualism reflects a commitment to empiricism, commitment to the idea that suggested evolutionary transitions should be represented by evidence. Saltational "punctuated equilibrium" postulates how speciation takes place, based not on empirical evidence but on negative evidence — gaps in the fossil record. "Punctuated equilibrium is unscaled, and by nature untestable. It hardly deserves recognition as a conjecture of "major importance for palaeontological theory and practice". Palaeontology, like other scientific disciplines, is dedicated to the principle of empirical testability: hypotheses that cannot be tested are of little value in science.

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●Rhodes replies — My recent article¹ showed that three of the four tenets of "pure gradualism" attributed to Darwin², were, in fact, repudiated by him and that his view of transmutation embraced the major tenets and consequences of what we now call "punctuated equilibrium."

With that, and with the forty-six paragraphs required to demonstrate it, Gingerich agrees. It is with only the penultimate

paragraph of my article that Dr Gingerich disagrees. Two questions are involved.

(1) Gingerich questions my description of punctuated equilibrium as a hypothesis of "major importance for palaeontological theory and practice". Webster defines a hypothesis as "a tentative assumption made in order to draw out and test its logical or empirical consequences". This is precisely what the hypothesis of punctuated equilibrium has done. It has provoked adulation and anger but it has also stimulated a remarkable flurry of detailed analytical studies designed to test the hypothesis. These range from fossil hominids³ to Ordovician trilobites⁴, Pennsylvanian gastropods⁵, Mesozoic⁶ and Cenozoic molluscs⁷⁻⁹, radiolarians¹⁰ and pollen¹¹. The stimulus provided by the hypothesis to the study of its possible consequences in living organisms has been no less fruitful, including, for example, the evolutionary relationships of frogs¹², mimetic butterflies¹³ and patterns of behaviour¹⁴. It continues to generate intense debate concerning the possibility of testing the hypothesis against the fossil record¹⁵ and the nature of the evolutionary process itself^{16,17}.

If the test of a "major hypothesis" is that it stimulates major debate and testing of its logical or empirical consequences, the hypothesis of punctuated equilibrium surely qualifies.

I do not argue here for the validity of punctuated equilibrium: I do not argue for its distinctiveness — or lack of it — within a continuum of populations: I do not argue for the adequacy of the fossil evidence available to validate the hypothesis: I do not argue that it meets the canon of "empirical testability". All of these deserve continuing discussion. But I do argue that punctuated equilibrium — whether true or false — is a "hypothesis of major importance" and that it has had a beneficial impact on the quality of recent palaeontological studies.

(2) Gingerich asks, "Which nuances [of punctuated equilibrium] were unanticipated by Darwin?" From a long list, I suggest the following: its relationship to the genetics of stasis and of punctuation¹⁸, morphological stasis and developmental constraints¹⁹, evolutionary models in relation to palaeoecology²⁰, stratigraphical correlation²¹, species selection²², mathematical models of evolutionary rates²³, selection of RNA molecules²⁴, phylogenetic divergence²⁵, the molecular basis of adaptation²⁶, and the evolution of communities²⁷. These topics, and many more studied from the viewpoint of punctuated equilibrium, have been the subject to recent papers.

Although Darwin could scarcely have been expected to anticipate such nuances of these, the remarkable thing is that he did recognize and accept the major components of the present hypothesis of punc-

tuated equilibrium. (So also did Simpson²⁸, whose contribution has been too little recognized in the current debate.) But to suggest that there was no nuance of punctuated equilibrium which was "unanticipated by Darwin" is to make an icon of Darwin and to adopt an extravagantly Whiggish view of the history of science of Darwin's particular contribution — great as that was.

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Seminal lymphocytes, plasma and AIDS

SIR — It has been suggested that cells or soluble agents in semen might induce immunosuppression following passage through rectal mucosal monolayers into lymphatics or blood vessels after injury during passive anal intercourse^{1,2}. And the relatively great thickness of the vaginal mucosa has been put forward to explain the relatively low incidence of acquired immune deficiency syndrome (AIDS) in US women not transfused or using drugs². Emphasizing the remarkable emperipoetic (Gr. *em*, in — *peri*, around — *poiesis*, wandering about)^{3,4} motility of small lymphocytes, others^{5,6} suggested that seminal lymphocytes infected with lymphotropic retroviruses might transfect AIDS relatively easily through the thin rectal mucosa, with or without injury. Passage through seems confirmed experimentally by finding anti-lymphocyte antibodies in healthy male rabbits given semen via anal catheterization from others with or without vasectomy⁷.

Through such emperipoetic lymphocyte transfection one might explain immune abnormalities in a woman routinely engaging in anal intercourse⁸, as well as the relatively high incidence of AIDS in Haitian women

Table 1 Lymphocytes and sperm in heterosexual male ejaculates

Male	Volume (ml)	Sperm ($\times 10^6$ per ml)	Leukocytes ($\times 10^6$ per ml)	Lymphocytes (per cent)	Lymphocytes ($\times 10^6$ per ml)	Lymphocytes (per ejaculate)
(1)	0.3	118	0.14	98	0.14	42,000
(2)	2.5	55	1.2	90	1.08	2,700,000
(3)	5.0	33	0.2	79	0.16	790,000
(4)	7.5	34	9.0	43	3.87	29,000,000
(5)	2.0	49	10.0	66	6.60	13,200,000
(6)	4.2	19	6.0	86	5.16	21,700,000
(7)	3.5	46	3.4	94	3.20	11,200,000
(8)	3.0	84	0.2	76	0.15	456,000
(9)	3.5	86	4.2	93	3.91	13,700,000
(10)	1.5	100	2.6	90	2.34	3,500,000
(11)	3.5	20	1.0	92	0.92	3,200,000
(12)	3.0	111	2.2	84	1.85	5,500,000
Average	(3.3)	(63)	(3.35)	(83)	(2.45)	(8,750,000)

Post-vasectomy						
(1)	2.0	0.0	0.56	77	0.43	860,000
(2)	3.0	0.0	0.06	88	0.05	158,000
(3)	2.5	0.0	0.90	62	0.56	1,400,000
(4)	4.0	1.5	0.08	93	0.07	298,000
(5)	2.5	0.0	0.06	96	0.06	144,000
(6)	3.0	0.0	0.08	94	0.08	226,000
(7)	3.0	0.0	0.40	95	0.30	1,140,000
(8)	2.5	2.2	0.05	75	0.04	94,000
(9)	1.5	0.0	0.80	86	0.69	1,030,000
Average	(2.7)		(0.33)	(85)	(0.25)	(594,000)

Ejaculates from heterosexual human males without and with vasectomy were studied by direct chamber counting and differential counts in stained smears. The specimens were supplied by unselected men consecutively studied at the SBMFC laboratory from 1 October to 31 December 1983 for infertility or for the effectiveness of vasectomy. Note that whether or not sperm are present, leukocytes are found constantly in semen. Of the seminal leukocytes, usually more than 83% (range 43–98%) are small lymphocytes, perhaps owing to their emperipoietic nature^{3,4}. The number of lymphocytes per ml of semen vary greatly (from 40,000 to 6,600,000) with averages of 0.25×10^6 in vasectomized males and 2.45×10^6 in males with vasa intact. Such variations might be owing to counting problems associated with attaining uniform dilutions, individual difficulties with collection of specimens or lack of selection with respect to age and health of the male population studied.

prone to use anal intercourse as a method of birth control⁹. To ascertain how many seminal lymphocytes might be involved in humans, we studied ejaculates from heterosexual males undergoing routine fertility studies, as shown in Table 1. The data indicate that semen normally contains relatively large numbers of small lymphocytes which could transfect lymphotropic virus or induce immuno-deficiency in the peresence or absence of sperm.

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SIR — Studies during recent years have convincingly demonstrated that human seminal plasma can impair the activity of many of the cells involved in the immune response and also interfere with the generation and activity of a number of soluble factors involved in the defence against infection. Thus small concentrations of seminal plasma (1 per cent volume or less)

are known to inhibit the *in vitro* response of lymphocytes to a variety of stimuli including lectins, alloantigens, sheep erythrocytes, hapten-sheep erythrocyte conjugates, bovine serum albumin and tetanus toxoid (see, for example, refs 1,2). Inhibitory effects have also been noted *in vivo*².

Evidence has recently emerged to suggest that some of the reported effects might be attributable to impairment of the function of accessory cells such as macrophages³. It seems that many properties of such cells are inhibited by small doses of seminal plasma, including their capacity to act as accessory cells in mitogen induced lymphocyte blastogenesis and their ability to phagocytose inert particles and generate reactive oxygen species following triggering with opsonized zymosan. The response of granulocytes to opsonized zymosan is likewise impaired³. More recently it has become apparent that seminal plasma drastically inhibits the activity of natural killer (NK) cells *in vitro* (K. J. and S. Szymaniec, in preparation). These observations are particularly relevant in view of the impaired NK cell activities observed in AIDS⁴ and the role such cells may play in tumour surveillance⁵ and in controlling B cell proliferation and differentiation⁶.

Others have found that seminal plasma may interfere with the activity of complement components (especially C1 and C3), inhibit the alternate complement pathway⁷ and block binding of IgG molecules to Fc receptors on cell membranes⁸.

The identity of the factor(s) involved remains to be established, although there is no shortage of possible candidates. It is

highly likely that the different effects observed are mediated by different components. Whatever the nature of the factors it is interesting to note that seminal plasma proteins do not readily cross the vaginal mucosa². In contrast the anal route is frequently used for administering substances that have a systemic effect.

The observed effects are undoubtedly relevant to a variety of clinical conditions, in addition to AIDS. These include chronic prostatitis, cancer of the prostate and cervix, and sexually transmitted diseases in general. In the latter context it is interesting to note that there are reports that human seminal plasma can inhibit both the antibody and cell mediated killing of *Neisseria gonorrhoeae* and *Escherichia coli*⁷. Further consideration of the possible clinical relevance of the wide ranging immunosuppressive effects of semen and seminal plasma is clearly warranted.

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Water pipe problem is solved

SIR — In places where the atmospheric humidity is high, condensate forms on smooth surfaces which have a temperature lower than that of the dew point. One such surface is the outside of horizontal water pipes. Gravity causes the condensate to drain and collect into globules on the undersurface of the pipes; when the suspended globules grow to a size governed by the surface tension and gravitational forces, they detach and fall. I have measured the approximately equal spacings between such globules on a 1½-inch pipe. They range from 1.4 to 2.0 cm with an averaged value of about 1.7 cm.

This phenomenon may be viewed as an example of Rayleigh-Taylor instability¹. As liquid collects on the underside of a horizontal surface we have the situation of a heavy fluid (water) above a lighter fluid (air). Then, the maximum wavelength, λ , consistent with stability is given by

$$\lambda = 2\pi \left[\frac{T}{g(\rho_L - \rho_G)} \right]^{1/2}$$

where T is the surface tension and g the acceleration due to gravity, and ρ_L and ρ_G the density of liquid and gas respectively. The numerical value for λ calculated for the air-water system is 1.73 cm. This is in excellent agreement with the averaged measured value of about 1.7 cm despite the fact that the pipe had a rather irregular outside surface which would result in considerable bias in the draining of the condensate.

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Does stress increase ecosystem diversity?

SIR — Peter D. Moore (*Nature* 306, 17; 1984) exposes the general state of confusion about diversity of communities. He reports various authors, concluding that a certain degree of stress may be a factor increasing diversity of a community and quotes several examples, and he implies that stress can take the form of a physical limitation to overall productivity or of disturbance. However, in every case, stress is used to mean an increase or decrease of habitat heterogeneity or of the community itself by the addition or elimination of other species, or physical factors, or by simulating their action. In a quoted experiment on diversity of algae (A) grazed upon by coral reef fish species (B) which in turn are hunted by predatory fish (C), manipulation of both B and C affects diversity of A. If C is absent, then overgrazing reduces A's diversity. If B and C are absent, then A diversity is higher than in the absence of C only, but lower than in the presence of both B and C. (In the last instance, results may be inconclusive as a downward trend in algae diversity was observed at the end of the experiment).

It becomes clear that the real system being studied is not an algae assemblage but a complex structure composed of non-equivalent elements of A, B and C which are linked by a number of functions of which trophic appears to be the most important. The whole structure has its own diversity, or species richness, and by removing some of the species, particularly those functionally most important, we decrease its overall diversity which affects other parts of the system in a variety of patterns. The reason for that, most probably, is that competition acquires the sole role in shaping species composition and abundance pattern. It is not, however, the fact of impact of predator on algal diversity that I question but the term "stress". The problem immediately arises if we try to estimate what is the stress of the combined system of A, B and C in which A manifested the highest diversity. And what would happen if one more trophic level is added? Perhaps the real application of stress would be removal of B and/or C from the complete system A,B,C. Moreover, if B is absent, then there would be no stress on A and maximum stress on C (hunger in this case). What seems to be the case is that the concept of stress is a derivative of the currently applied notion of the community. Depending on one's view, the stress is either present or absent. As the community unit is as subjective in most studies as in this discussion, too great a relativity of the stress makes it irrelevant as a theoretical concept. It leads to paradoxes where sometimes a keystone species increases diversity and sometimes it is necessary to speak of a "reverse keystone species effect" (Hixon, M.A. & Brostoff, W.N. *Science* 220, 511; 1983).

In short, the "stress concept" says that diversity increases (decreases) if overall diversity of the system increases (decreases) by adding species: grazers and predators in this case. The new wording touches upon a circular argument. This mistake is more difficult to notice when a non-functional fragment of community is analysed in separation from the functional whole.

It seems that this, partly semantic, confusion results from our imperfect concepts of ecological systems (communities) stemming from our lack of a coherent theory of ecosystem organization and it leads to a misinterpretation of observed phenomena (Kolasa, J. & Biesiadka, E. *Acta biotheoretica*; submitted).

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How do you spell DNA?

SIR — In a provocative column, you discussed the theory and practice of artificial intelligence¹, using spelling correction as an example of a task in which automated computer procedures should be of great value. In the past ten years, a number of tools have been developed to solve similar problems in molecular biology.

The usual connection between molecular biology and language is made by drawing parallels between the bases of DNA and the letters of the alphabet, between codons and words, and between proteins and sentences. Expanding on this analogy, mutations in DNA sequences can be thought of as spelling errors, creating new sequences from those already in existence. The processes of evolution, whether of DNA or language, select which of these new sequences or words are to survive.

What then can computer programs tell us about these molecular spelling errors? One approach, pioneered by Needleman and Wunsch and elaborated by Sankoff, Sellers and others, maximizes matches, subtracting a weighted sum of mismatches and insertion/deletions, between two DNA or protein sequences. For example, AATCAG and ATTCG might be related by

A A T C A G
A T T C * G

where there is one point mutation and one insertion/deletion. Two sequences of length N have of the order of 2^{2N} possible relationships. For $N = 1,000$, this number is approximately 10^{600} so that an exhaustive search is impossible. Nonetheless, rigorous algorithms and programs exist to locate rapidly the optimal relationships for sequences of 1,000 or more bases. Many modifications and improvements exist. Long insertions/deletions can be weighted according to length. Other mutational events, such as inversions, are being incorporated into these rapid comparison algorithms.

These algorithms can be extended in a novel way to predict RNA secondary structure; the base-paired regions correspond to

matches in the sequence comparison algorithms. Indeed, these basic methods have been adapted and applied to speech recognition, geological strata, handwriting recognition, bird song and gas chromatography. Many such applications and associated theory appear in a recent book edited by D. Sankoff and J.B. Kruskal². In addition to spelling correction, the analogue to a dictionary search for a word is the comparison of a new sequence to an existing DNA or protein data base. These searches locate library sequences with regions of similarity to the new sequence, combining the concepts of dictionary and imperfect spelling. New tests are being devised to estimate the statistical significance of the similarities. Methods to perform these large searches have been developed, and were successfully applied in the recent discovery of sequence similarity between the transforming protein of a primate sarcoma virus and a platelet-derived growth factor^{3,4}. Another recent computer finding indicates that an oncogene product appears to have arisen as a result of recombination of two unrelated cellular genes⁵.

One of the most intriguing areas of DNA sequence analysis parallels the concept of language interpretation. Patterns such as repetitive DNA are frequently noticed before their meaning is understood. An important case is the search for promoter sequences in *Escherichia coli*⁶. DNA upstream from *E. coli* coding regions is presumed to contain base sequences that spell "begin transcription". These are searches for patterns of unknown composition and length which we know must occur, however imperfectly, within specific regions of DNA. Text editors, even those equipped to search for regular expression patterns⁷, are not adequate to this task. If useful and rigorous algorithms are developed for these tasks of locating imprecise words of unknown spelling, new and nontrivial insights could result.

New techniques of pattern recognition in DNA and protein sequences are resulting from creating and applying concepts of mathematics, statistics and computer science appropriate to specific questions of molecular biology. As often happens in science, these methods may turn out to have broad applicability.

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Organic metals

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The field of quasi-one-dimensional organic metals has emerged over the past decade as one of the most dynamic and fruitful areas of research. These new materials have most unusual properties and great technological potential. They provide a unique meeting ground for organic chemists, physical chemists and solid state physicists.

ALTHOUGH most organic solids are electrical insulators [room temperature conductivity, σ_{rt} , 10^{-9} – 10^{-14} ($\Omega \text{ cm}$) $^{-1}$], the possibility that organic solids might exhibit high electrical conductivity comparable with that of metals was suggested at least half a century ago^{1,2}. Now there are two classes of organic metals—charge-transfer complexes and polymeric hydrocarbons.

What follows is mostly concerned with charge-transfer complexes, which have been studied extensively since the beginning of the century^{3,4}. A stable charge-transfer complex is usually formed by the partial transfer of an electron from a donor to an acceptor molecule forming an ionic crystal of sorts in which either the cations, or the anions, or both, will be chemical groups of some complexity. Familiar donors include alkali metals, amines, or electron-rich alkenes, heterocycles or aromatic molecules; among familiar acceptors are halogen atoms, quinones, or electron-deficient alkenes, heterocycles or aromatic molecules. The donor and acceptor molecules stack alternately face-to-face within the crystal lattice of these complexes (Fig. 1a).

The first evidence that crystalline charge-transfer complexes could be electrically conducting dates back to the early 1960s when Melby and co-workers at Dupont described the synthesis and properties of a new powerful electron acceptor, 7,7,8,8-tetracyano-*p*-quinodimethane (TCNQ) (structure 1)^{5–7}. A great many complexes of TCNQ with various donors were found to be semiconductors [$\sigma_{\text{rt}} \approx 10^{-5}$ ($\Omega \text{ cm}$) $^{-1}$] and quinolinium-TCNQ was the best organic conductor known at the time. In 1964, Little proposed a model for a high-temperature organic superconductor involving conduction along linear hydrocarbon chains surrounded by polarizable dye molecules⁸, prompting an upsurge of research centering at first on new TCNQ salts. It quickly became apparent that high conductivity is associated with crystal structures in which the donor and acceptor molecules formed segregated stacks (Fig. 1b), with considerable π -electron overlap and delocalization along these one-dimensional stacks and, consequently anisotropic conductivity. The preparation⁹ of a new donor, tetrathiafulvalene (TTF) (structure 2) in 1970 led, two years later, to the discovery that the chloride salt of TTF had high conductivity¹⁰ [σ_{rt} , 0.2 ($\Omega \text{ cm}$) $^{-1}$] and in 1973 the complexes of TTF^{11,12} and *N*-methylphenazinium (34)¹³ with TCNQ were both found to be metallic at room temperature [σ_{rt} 500 and 200 ($\Omega \text{ cm}$) $^{-1}$ respectively]. Organic metals had become a reality.

During this period, there has been equal interest in polymeric hydrocarbons with extensive π -electron delocalization, where there is the possibility of a narrow conduction band, derived from the π -orbitals. Polyacetylene has received most attention; in the pure state the material is an insulator [σ_{rt} 10^{-9} ($\Omega \text{ cm}$) $^{-1}$] with a relatively large band gap, but Heeger, MacDiarmid and co-workers^{14–16} showed in 1977 that doping with strong oxidizing and reducing agents increases the conductivity of polyacetylene dramatically to $\sigma_{\text{rt}} \approx 500$ ($\Omega \text{ cm}$) $^{-1}$, since when there has been much work on polyacetylene and its derivatives¹⁷. Two other related classes of one-dimensional conductors include polymers

of the main group elements, for example, (SN)_x (ref. 18) and linear-chain chelated transition metal compounds where intra-chain overlap involves the ligand π -system as well as metal-metal interactions¹⁹. For more information on such materials see refs 20–31.

Physical concepts

Because of their crystal structure (Fig. 1b), conducting organic charge-transfer complexes may be likened to arrays of one-dimensional molecules with more than the complement of electrons required for valence bonding. In principle, the extra electrons will partially fill a conduction band whose width is determined by the interactions between neighbours. The behaviour of such a system was discussed in the mid-1950s when Peierls³² pointed out that, at low temperature, a quasi-one-dimensional metal could not sustain long range order but would be unstable with respect to lattice distortions. In the simplest terms, the conducting chain would be stretched in one region and contracted in another, so that the conducting electrons become localized, with a filled electron band at a lower energy and an empty band at a higher energy. A variation of charge density in phase with the lattice distortion, such as this, is known as a charge density wave, and the associated energy gap results in an insulating, or at best, a semiconducting solid. As part of an explanation of superconductivity, however, Fröhlich pointed out in 1954 that if charge density waves were coupled to the vibration of the underlying lattice, a charge density wave could travel freely through the crystal, affording a superconducting rather than an insulating state. When the number of electrons in a chain is commensurate with the number of lattice sites, a metal-to-insulator transition can be expected to occur readily as the electron charge density wave becomes static because of the high potential energy which must be overcome in order for electrons to move to new equilibrium positions along the chain. On the other hand, a charge density wave incommensurate with the lattice will not be locked to the lattice and will be free to translate to new positions, so acting as a charge carrier, without adversely affecting the energetics of its relationship to the lattice. Thus a mechanism for high conductivity is available and for a truly incommensurate system a superconducting state will exist³³. A spin modulation, without a lattice modulation, provides another possible instability for one-dimensional conductors. Here, the electron spin density distorts along the chain, in the extreme case giving an anti-ferromagnetic arrangement of spins. Lattice defects and impurities can also lead to random electrostatic potentials that will tend to pin the charge density wave to the underlying lattice and favour Peierls distortions.

Tetrathiafulvalene-tetracyano-quinodimethane

Since the discovery of TTF-TCNQ¹¹ there has been unabated interest in the design, synthesis and properties of new highly conducting charge transfer complexes and many derivatives and analogues are known. Nonetheless TTF-TCNQ remains one of

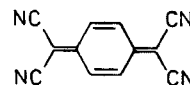
the most extensively studied, partly because it is relatively easy to grow single crystals of good size and quality, and also because the complex exhibits most of the remarkable structural, electrical, magnetic and optical properties characteristic of an organic metal.

The crystal structure of TTF-TCNQ consists of TTF and TCNQ molecules uniformly spaced along the *b* axis in interlocking, segregated stacks (Fig. 2)^{34,35}. Within these segregated donor and acceptor columns, the molecules do not lie directly on top of one another; there is a lateral displacement giving 'ring-over-bond' overlap—the exocyclic C=C double bond lies over the ring of the molecule adjacent to it in the stack. The conductivity of TTF-TCNQ rises 20 fold from 500 ($\Omega\text{ cm}$)⁻¹ at 293 K to $>10^4$ ($\Omega\text{ cm}$)⁻¹ at 59 K (ref. 12), but on further cooling three successive phase transitions occur at 53, 47 and 38 K leading eventually to an insulating state with three-dimensional order. The results of numerous studies at temperatures below the metal-semiconductor transition have been interpreted in terms of a Peierls distortion³⁶⁻⁴². The electronic properties of TTF-TCNQ are highly anisotropic. Calculations from the value of the Peierls distortion wave vector³⁶⁻³⁹ and more recently from IR data⁴³ show that charge transfer from TTF to TCNQ is incomplete, with 0.59 electrons in the TCNQ band, so with both bands partially filled, both stacks contribute to the metallic conductivity. Details of the mechanism of conductivity are still the subject of considerable debate. Initially there was support for collective effects involving a sliding charge density wave contribution⁴⁴, while other workers proposed single-particle conductivity⁴⁵, and it is possible that both mechanisms are important depending on temperature⁴⁶.

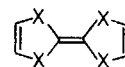
So what makes TTF and TCNQ stand out over traditional donors and acceptors? Certain key features of the molecules were quickly appreciated: both molecules are of similar size; both are planar with π -delocalization extending throughout the molecule; they both have the same high degree of symmetry (D_{2h}); and the ionization potential/electron affinity values for the pair favour incomplete charge transfer. Considerable research effort has been directed towards the synthesis of new donors and acceptors to test the importance of these factors, and the number of organic compounds reported with high conductivity continues to grow rapidly. The goal has mostly been the stabilization of the metallic state and the attainment of organic superconductivity. From the plethora of complexes that have been prepared and theories that have been advanced, we will now select those areas that have proved most productive or are currently captivating a significant proportion of workers in this field.

New electron donors

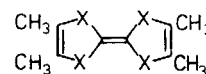
Synthetic chemists have given much attention to new donors containing the 1,3-dithiole ring system of TTF (see ref. 47). First to be studied were derivatives with an extended σ -bond framework, for example, tetramethyl-TTF (structure 5)⁴⁸ and hexamethylene-TTF (structure 7)⁴⁹, or an extended π -orbital system, for example, dibenzo-TTF (structure 10)⁵⁰. The next logical, but chemically demanding, step involved replacement of sulphur with selenium, for example, TSF (structure 3)⁵¹, TMTSF (structure 6)⁵², HMTSF (structure 8)^{53,54}, and recently DBTSF (structure 11)^{55,56} have been prepared. In general the TCNQ complexes of the tetraselenafulvalenes show increased stability of the metallic state compared with that of their TTF counterparts and the donor stack dominates the transport properties due to the presence of selenium *d* orbitals. The steric similarity between TTF-TCNQ and TSF-TCNQ and the fact that these materials are isostructural, has enabled extensive systematic studies⁵⁷ to be made of the alloy system (TSF)_x(TTF)_{1-x}-TCNQ. Derivatives of TTeF (structure 4) have been long awaited. It was anticipated that there would be a further stabilization of the metallic state in salts derived from these compounds due to increased bandwidth and intrachain couplings. The first derivatives, HMTTeF (structure 9)⁵⁸ and DBTTeF



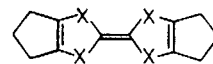
(1) 7,7,8,8-Tetracyano-*p*-quinodimethane (TCNQ)



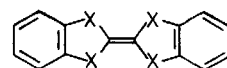
- (2) Tetrathiafulvalene (X = S, TTF)
 (3) Tetraselenafulvalene (X = Se, TSF)
 (4) Tetrallurafulvalene (X = Te, TTeF)



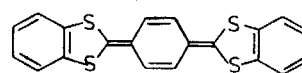
- (5) Tetramethyltetrathiafulvalene (X = S, TMTTF)
 (6) Tetramethyltetraselenafulvalene (X = Se, TMTSF)



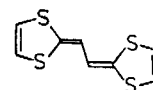
- (7) Hexamethylenetetrafulvalene (X = S, HMTTF)
 (8) Hexamethylenetetraselenafulvalene (X = Se, HMTSF)
 (9) Hexamethylenetetratellurafulvalene (X = Te, HMTTeF)



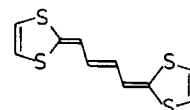
- (10) Dibenzotetrathiafulvalene (X = S, DBTTF)
 (11) Dibenzotetraselenafulvalene (X = Se, DBTSF)
 (12) Dibenzotetratellurafulvalene (X = Te, DBTTeF)



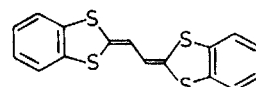
(13) Cyclohexa-2,5-diene-1,4-diylidene-bis(1,3-benzodithiole)



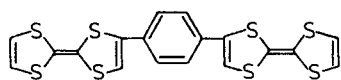
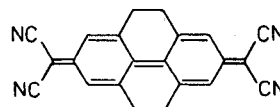
(14) Ethanediylidene-2,2'-bis(1,3-dithiole) (EDBT)



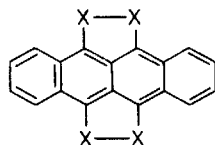
(15) 1,4-Butenediylidene-2,2'-bis(1,3-dithiole) (BDBDT)



(16) Bis-(1,3-benzodithiole-2-ylidene)ethane

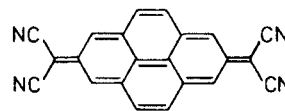
(17) *p*-Phenylenebistetrathiafulvalene

(29) 13,13,14,14-Tetracyano-4,5,9,10-tetrahydropyrenoquinodimethane (TCNTHPQ)

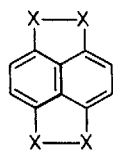


(18) Tetrathiatetracene (X = S, TTT)

(19) Tetraselenatetracene (X = Se, TST)



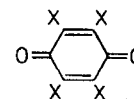
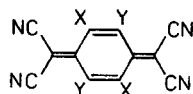
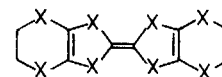
(30) 13,13,14,14-Tetracyanopyreno-2,7-quinodimethane (TCNPQ)



(20) Tetrathianaphthacene (X = S, TTN)

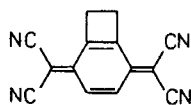
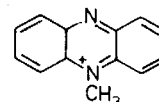
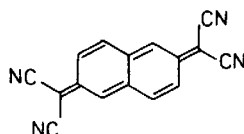
(21) Tetraselenanaphthacene (X = Se, TSN)

(22) Tetratelluranaphthacene (X = Te, TTeN)

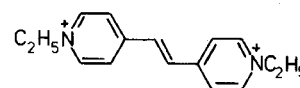
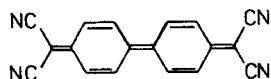
(31) Tetrahalo-*p*-benzoquinone (X = Halogen)(23) Tetrafluorotetracyano-*p*-quinodimethane (X = Y = F)(24) 2,5-Dibenzyltetracyano-*p*-quinodimethane (X = CH₂Ph, Y = H)(25) 2,5-Dibromotetracyano-*p*-quinodimethane (X = Br, Y = H)

(32) Bis(ethylenedithio)tetrathiafulvalene (X = S, BEDT-TTF)

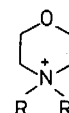
(33) Bis(ethylenedithio)tetraselenafulvalene (X = Se, BEDT-TSF)

(26) 2,3-Dimethylenetetracyano-*p*-quinodimethane(34) *N*-Methylphenazinium (NMP)

(27) 11,11,12,12-Tetracyanonaphtho-2,6-quinodimethane (TNAP)

(35) 1,2-Di(*N*-ethyl-4-pyridinium-ethene) (DEPE)

(28) 13,13,14,14-Tetracyanodiphenoquinodimethane (TCNDQ)

(36) 4-Methyl-4-ethylmorpholinium (R = CH₃, R' = C₂H₅, MEM)(37) 4,4-Diethylmorpholinium (R = R' = C₂H₅, DEM)

(structure 12)⁵⁹ were both reported in 1982. A 1:1 TCNQ complex of HMTTeF has powder conductivity [$\sigma_{\pi} \approx 1 (\Omega \text{ cm})^{-1}$]⁵⁸ comparable with that of TTF-TCNQ. X-ray analysis of HMTTeF reveals that the structure is remarkably similar to HMTSF in molecular dimensions, yet strikingly different in the mode of packing, in that HMTTeF shows no tendency to form uniform stacks⁶⁰. Further studies on complexes of these tellurium donors are expected.

Donor molecules with extended conjugation between two 1,3-dithiole rings and more widely spaced sites of maximum spin have also been studied. The molecules (structures 13⁶¹, 14⁶², 15⁶³ and 16^{64,65}) are known, and a highly conducting TCNQ complex of (16) has been reported⁶⁵. Wudl⁶⁶ has emphasized that in highly conducting complexes, Peierls distortions which break the metallic state should be disfavoured by the introduction of disorder which in effect increases the dimensionality of the solid. With this in mind donor (structure 17) was prepared and reported to form a metallic 1:1 complex with TCNQ⁶⁷; more detailed studies of this complex would seem worthwhile.

One-dimensional conductors based on planar aromatic donors containing several S, Se or Te atoms at the periphery of the molecules have also received continued attention since early reports that tetrathiatetracene, TTT (structure 18), formed cation radical salts with a range of anions⁶⁸. The iodide salt TTT-I_{1.5} is very highly conducting [σ_{π} 600–1200 ($\Omega \text{ cm})^{-1}$]⁶⁹ and subsequently numerous TTT halides, of a range of stoichiometries, have been prepared and their metallic properties studied^{70–73}. Electrocrystallization techniques have proved most valuable in providing salts of new stoichiometries⁷⁴. These compounds offer an unusually good opportunity for studying the subtle effects of the donor-to-acceptor ratio on properties. TTT forms a 1:1 and a 1:2 complex with TCNQ; the latter is the more highly conducting at room temperature⁶⁸, a fact consistent with TTT being fully ionized. Tetrathianaphthalene, TTN (structure 20), has the sulphur atoms free from hindering peri-hydrogens and hence better positioned for strong intrachain coupling. However, TTN is a much poorer donor than TTF or TTT, and TTN-TCNQ is metallic over only a narrow temperature range⁷⁵. Halides of tetraselenatetracene, TST (structure 19) have also been characterized⁷⁶. Recently the preparation of TSN (structure 21)⁷⁷, the X-ray structure of TTeN (structure 22)⁷⁸ and metallic complexes of tetrathia- and tetraselena-anthracenes have been described^{79,80}.

New electron acceptors

Over the past decade, fewer publications have been concerned with the study of new acceptors than of new donors, although some important advances have been made. This is partly because the synthesis of many TCNQ derivatives is not easy to accomplish, although in an important early paper Wheland and Martin characterized 21 derivatives⁸¹, and their complexes with TTF and some other donors were obtained^{82,83}. These TCNQ derivatives offer a spectrum of varying electron affinities and accordingly their complexes provide important experimental evidence regarding the effect of band filling on transport properties. In general, substitution on the basic TCNQ skeleton results in complexes that are less conducting than those of TCNQ itself. For a striking example, compare salts of TCNQ and tetrafluoro-TCNQ (structure 23). Although HMTSF-TCNQ and HMTSF-TCNQF₄ are isomorphous, the former is one of the most highly conducting organic salts known [σ_{π} 1,500 ($\Omega \text{ cm})^{-1}$]⁵⁴ whereas the latter has a room temperature conductivity almost nine orders of magnitude lower [σ_{π} 10⁻⁶ ($\Omega \text{ cm})^{-1}$]⁸⁴. The key to this lies in the degree of charge transfer. For HMTSF-TCNQ charge transfer is partial (0.74 electron molecule, compared with 0.59 electron molecule for TTF-TCNQ), whereas for HMTSF-TCNQF₄ charge transfer is complete, because TCNQF₄ is a far stronger electron acceptor than TCNQ, and a fully ionic Mott-Hubbard insulator results^{27,85}. A similar case⁸⁶ obtains with the complexes of HMTTF and TCNQ and TCNQF₄.

Di-substituted TCNQ derivatives have the substituents in the

<i>a</i> DADA	<i>b</i> DADA
ADAD	DADA
DADA	DADA
ADAD	DADA
DADA	DADA

Fig. 1 *a*, Mixed donor-acceptor stacks. *b*, Segregated donor and acceptor stacks.

2,5-positions, so Japanese workers broke new ground with their recent synthesis of cyclobutane-fused TCNQs, for example, (structure 26)⁷. The cyclobutane ring has little effect on the electronic state of the parent TCNQ, but should influence the interplanar and interstack spacings in the segregated columns in the complexes. A 1:1 complex of structure 26 with TTF is metallic⁸⁷ but no structural data have been reported yet.

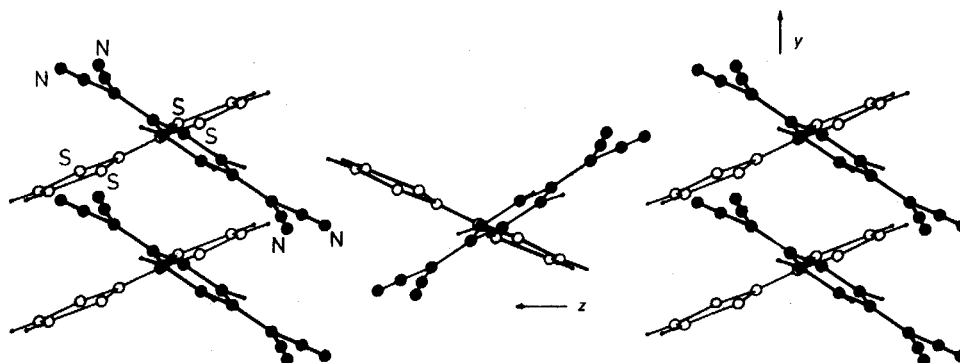
A particularly interesting molecule is 2,5-dibenzyl-TCNQ (structure 24) with two weakly electron denoting benzyl groups attached to the strongly electron accepting TCNQ framework⁸⁸. This is considered to be a prototype for a new class of organic metals where donor and acceptor moieties are present in one molecule and are thus in a prefixed stoichiometric ratio. While, predictably, structure 24 is not a conductor, elaboration of the units of this compound with alteration of their donor-acceptor properties offers a unique chance to manipulate electron transfer within an organic solid. In this regard, the X-ray crystal structure of structure 24 is encouraging⁸⁸.

As mentioned previously, the X-ray crystal structure of TTF-TCNQ shows ring-over-bond overlap along both the donor and acceptor stacks. Calculations suggest that the TCNQ stack is making its maximum possible contribution to the conductivity of TTF-TCNQ^{89–91}, although the contribution of the TTF stack would be increased if the TTF molecules stacked directly on top of each other, as is found for the highly conducting TTF-halides⁹². It was hoped that changing the size and shape of the acceptor might provide TTF complexes where both stacks were making their maximum contributions to the conductivity. It is known^{93,94} that in TCNQ anions the excess electron density resides on the terminal dicyanomethylene groups, so this functional group has been retained in new acceptors with extended π systems. Accordingly TNAP (structure 27) was synthesized and although metallic complexes were obtained with TTF⁹⁵ and HMTSF⁹⁶ success was limited partly because good crystals were difficult to obtain. The highly symmetrical compound TCNDQ (structure 28) was also of interest because its larger size compared with TCNQ should reduce coulombic repulsions in its dianion. However, initial attempts to synthesize structure 28 failed, leading only to polymeric material⁹⁷, although both the dianion TCNDQ²⁻ and the radical anion TCNDQ⁻ were studied by other workers⁹⁸. A charge transfer complex of TCNDQ with TTF has now been prepared but very little solid state data have been reported⁹⁹.

The next logical step was to prepare the pyrene derivatives TCNTHPQ (structure 29) and TCNPQ (structure 30), bridged analogues of TCNDQ. Compound 29, like 28, undergoes two reversible reductions to the radical anion and dianion as studied by cyclic voltammetry^{99,100}. The complex TMTTF-TCNTHPQ was found¹⁰⁰ to have a room temperature conductivity of 0.2 ($\Omega \text{ cm})^{-1}$. Although TCNPQ (structure 30) is a better acceptor than TCNQ, the electron affinity of the former^{101,102} is still within the range that should favour incomplete charge transfer, and hence high conductivity as with donors of TTF type²⁷. However, such complexes have not been reported, possibly because these new acceptors are simply too large to form stable crystalline complexes. So, overall, the results of some extensive synthetic work directed towards extended TCNQ derivatives have been disappointing and early hopes that this approach would afford conducting complexes have not been realized.

A different and more successful approach to the choice of new acceptors has been used by Torrance and co-workers,

Fig. 2 X-ray crystal structure of TTF-TCNQ (from ref. 35).



following guidelines that stress the importance of stacking, stoichiometry and degree of charge transfer when designing complexes intended to be metallic²⁷. Sandman¹⁰³ and Mayerle *et al.*¹⁰⁴ put forward two theories concerning segregated versus mixed stacking and the idea was introduced that segregated stacking would be favoured when the frontier orbitals of the donors and acceptors had the opposite symmetry on inversion, as is the case with TTF-TCNQ and NMP-TCNQ (structure 33). Accordingly, tetrahalo-*p*-benzoquinones (structure 31) were chosen as acceptors and found to yield a variety of complexes with TTF and TMTTF some of which were, indeed, metallic at room temperature¹⁰⁵. This was an important milestone as these materials represented the first highly-conducting complexes that did not contain TCNQ, or a close derivative, as the acceptor. TMTTF-bromanil (structure 31, X = Br) has been studied in detail, and a comparison of its X-ray structure with that of TMTTF-TCNQ reveals striking structural similarities¹⁰⁶. The overlap and interplanar spacings of the TMTTF molecules are virtually identical in the two structures and ring-exocyclic double bond overlap, typical of TCNQ, is also observed within the bromanil stack. So although the TCNQ and bromanil molecules are considerably different in many respects, the essential features common to both acceptors (the quinonoid ring structure with the same nodal properties of the LUMO) appear to be the driving force for the analogous molecular overlap. The key difference between the two structures is the 0.1 Å greater interplanar spacing for the bromanil stack, relative to the TCNQ stack; this poorer electronic overlap in the former, probably caused by the bulk of the bromine atoms, may well be responsible for the somewhat lower conductivity of TMTTF-bromanil compared with TMTTF-TCNQ. In accord with this the room temperature conductivity for TMTTF-chloranil (structure 31, X = Cl) is slightly higher than that for TMTTF-bromanil¹⁰⁷. Vibrational spectra of the bromanil and chloranil complexes confirm partial charge transfer, but some important properties of TMTTF-bromanil are very different from those of TMTTF-TCNQ. Whereas in TMTTF-TCNQ the conductivity and magnetic susceptibility both become thermally activated below the Peierls transition (as is usual for TTF-TCNQ type salts), with TMTTF-bromanil the conductivity is already activated at room temperature, well above the transition temperature (75 K) in the susceptibility. So TMTTF-bromanil should be viewed as a magnetic semiconductor.

Data have recently been published on a 1:1 complex of TTF and *p*-dinitrobenzene (DNB)¹⁰⁸. The latter was chosen as an acceptor because it resembles TCNQ in two important ways: both molecules are of similar size and both have D_{2h} symmetry. Also, the electronegativity of the NO_2 group is known to be similar to that of the =C(CN)_2 group, so that both acceptors might have similar spin and charge distribution in their radical anions. However, TTF-DNB is a neutral complex which is an insulator at room temperature [$\sigma = 2.5 \times 10^{-7} (\Omega \text{ cm})^{-1}$] and the X-ray crystal structure shows mixed donor-acceptor stacks, unusual for a TTF complex. Nonetheless, some mixed stack complexes of this type have special interest, as Torrance has found that, under pressure, some of them undergo a reversible electronic phase transition to an ionic ground state¹⁰⁹. This

provides the first observation of a neutral-to-ionic transition in any kind of material.

Organic superconductors

The most exciting advances of the past few years have come from studies of TMTSF salts. In 1978, the complex TMTSF-2,5-dimethyl-TCNQ was found to be the first organic compound in which a truly conducting state is stabilized under pressure down to 1 K; at ambient pressure a sharp metal-to-insulator transition occurs at 42 K^{110,111}. Two theories were advanced to explain the low-temperature conductivity of TMTSF-DMTCNQ. The Fermi surface may be semimetallic due to interchain hybridization making a quasi-two-dimensional solid¹¹² or, on the other hand, there may be gradual growth at low temperatures of a superconducting pairing between electron states¹¹¹ (see below). These results stimulated the preparation of a range of cation radical salts, $(\text{TMTSF})_2\text{X}$ with inorganic anions (X = NO_3 , BF_4 , PF_6 , AsF_6 , SbF_6 , TaF_6 , ClO_4 and so on). In 1980 Bechgaard and co-workers reported that $(\text{TMTSF})_2\text{PF}_6$ showed superconductivity at 1 K under 1,200 atmospheres pressure¹¹³. Under sufficiently large pressure, superconductivity has been observed in most members of the series, with $(\text{TMTSF})_2\text{ClO}_4$ exhibiting superconductivity [$\sigma > 10^5 (\Omega \text{ cm})^{-1}$] at ambient pressure down to 1.4 K¹¹⁴. The essential structural feature of the isomorphous $(\text{TMTSF})_2\text{X}$ salts is a zig-zag stacking arrangement of TMTSF molecules in sheets separated by anions, with intra- and inter-stack contacts of selenium atoms^{115,116} (Fig. 3). The structure of the AsF_6 salt has been examined by Wudl who points to the presence of a chain of selenium atoms involving two stacks of TMTSF molecules, and clustering of selenium atoms, indicating the pseudo-two-dimensional nature of the salt¹¹⁷. From further consideration of the X-ray structure it seems clear that the conductivity cannot arise from carbon-carbon π orbital overlap, but must be derived entirely from homoatomic overlap of the seleniums. Thus the anions have a negligible, or at most subsidiary, role in the conduction process, although they influence other properties of the salts by controlling band filling and lattice parameters¹¹⁷.

The theory of superconductivity in metals, based on highly coordinated motion of electron pairs (Cooper pairs) rather than individual valence electrons, was formulated in 1957¹¹⁸. There is now considerable experimental evidence that, for $(\text{TMTSF})_2\text{X}$ salts, Cooper pairing takes place along the donor chains at temperatures perhaps as high as 30 K, well above the onset of bulk superconductivity. A striking feature of this family of salts is the absence of charge density wave instabilities (in contrast to TTF-TCNQ); instead, spin density wave instabilities dominate the low-temperature properties¹¹⁹; this can be explained by a "spin-charge separation" and "charge-localization hypothesis" developed by Wudl¹¹⁷. Essentially this hypothesis is based on two premises. First, that there is inhomogeneous charge and spin distribution in TMTSF due to mismatch in C-Se orbital size, thus reducing conjugation within the molecule and, second, that the π molecular orbital of the TMTSF radical cation is extensively polarized in one crystallographic direction by the adjacent anions. This hypothesis finds support in a

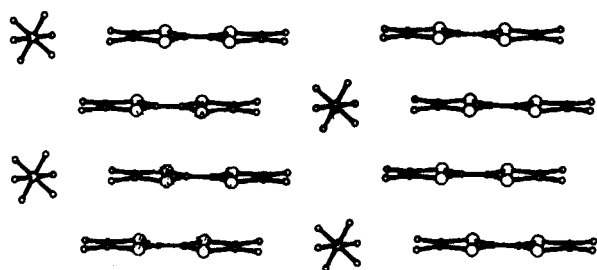


Fig. 3 X-ray crystal structure of $(\text{TMTSF})_2\text{PF}_6$ viewed in the plane of the TMTSF molecules (from ref. 116).

comparison between the salts $(\text{TMTSF})_2\text{X}$ and $(\text{TMTTF})_2\text{X}$: both families are isostructural, but the latter compounds have lower conductivities [σ_{rt} 50–300 compared with $500 (\Omega \text{ cm})^{-1}$] and undergo metal-to-insulator transitions at higher temperatures than their selenium counterparts^{119,120}. Nonetheless, some members of the $(\text{TMTTF})_2\text{X}$ series may undergo superconducting transitions under very high pressures¹²⁰. (For a discussion of the physics of these materials see ref. 121.)

Recent studies by Japanese workers^{122,123} on the new donor BEDT-TTF (32) are relevant here. The most unusual complex of structure 32 reported so far is the perchlorate salt $(\text{BEDT-TTF})_2\text{ClO}_4(1,1,2\text{-trichloroethane})_{0.5}$. This salt has aroused special interest as it is metallic over the temperature range 300–1.4 K, with no evidence of a metal-to-semiconductor transition. This suppression of the Peierls distortion is a major step forward on the road to designing new organic superconductors. Also, the salt shows two-dimensional metallic conduction over the same temperature range. The reason for this anisotropy is quite clear from the X-ray crystal structure¹²⁴. Unlike other organic metals, $(\text{BEDT-TTF})_2\text{ClO}_4(1,1,2\text{-trichloroethane})_{0.5}$ shows poor intermolecular overlap; the donor molecules are not stacked face-to-face, but instead are arranged side-by-side. The conduction pathway is considered to be derived principally from close intermolecular contacts of the sulphur atoms¹²⁴, akin to the selenium overlap in $(\text{TMTSF})_2\text{X}$ ¹¹⁷. Note that the importance of close intermolecular contact between chalcogen atoms in determining the conduction pathway has also been stressed by Underhill and co-workers for a metallic platinum complex, $\text{Li}_{0.75}[\text{Pt}(\text{C}_4\text{N}_2\text{S}_2)_2] \cdot 2\text{H}_2\text{O}$ (refs 125, 126). The selenium analogue (structure 33) has also been prepared and found to afford metallic complexes with ReO_4 and ClO_4 anions, and these await further study¹²⁷.

The characterization of these new materials is a significant advance that has been made possible by the extensive work on TTF-TCNQ and its analogues carried out during the 1970s. The same inorganic anions of the superconducting Bechgaard salts have been used to form radical cation salts of polycyclic aromatic hydrocarbons. Some of these salts, generated electrochemically, are also metallic at room temperature^{128,129}. The most highly conducting of these is the mixed system $(\text{perylene})_2(\text{AsF}_6)_{0.75}(\text{PF}_6)_{0.35}(\text{CH}_2\text{Cl}_2)_{0.85}$ which is metallic [σ_{max} $1,200 (\Omega \text{ cm})^{-1}$] down to 200 K¹³⁰. All salts of this family show a common structural feature of arene stacks with short intrastack distances between the molecular planes, and anions located in channels between the stacks¹²⁸.

TCNQ complexes with nitrogen heterocycles

Complexes of TCNQ with nitrogen heterocycles continue to attract attention and afford valuable information, although they have been overshadowed by complexes of the TTF-TCNQ and $(\text{TMTSF})_2\text{X}$ families. Three heterocycles can be singled out: phenazine, bipyridine and morpholine. The structural variation found in phenazine-TCNQ complexes is remarkable. *N*-methylphenazine-TCNQ (NMP-TCNQ) (structure 34), one of the first good organic conductors to be studied extensively¹³¹, forms segregated, regular stacks¹³², while *N*-ethylphenazinium-TCNQ

(NEP-TCNQ) is an insulator forming mixed donor-acceptor stacks. The X-ray structure of NEP-TCNQ is interesting because it shows long σ bonds linking TCNQ^- anions in adjacent stacks to form a spin-paired, diamagnetic ground state^{133,134}. Breaking of this weak σ bond leads to two TCNQ radical anions in close proximity and hence novel magnetic properties¹³⁴. Mixed stacking is also observed in the unsubstituted phenazine-TCNQ¹³⁵, and in the TCNQ^{136} and TCNQF_4 ¹³⁷ complexes of 5,10-dihydro-5,10-dimethylphenazine; magnetic studies show that this last complex provides one of the first truly ionic systems¹³⁷. Unusual ...AADDAAADD... dimerized columns found in 5-(1-butyl)phenazinium-TCNQF₄ provide a further structural variation^{138,139}. The X-ray structures of the 2:3 complexes, $(\text{NEP})_2(\text{TCNQ})_3$ (ref. 140) and $(N\text{-propylphenazinium})_2(\text{TCNQ})_3$ (ref. 141) show mixed stacks of phenazinium and TCNQ dimers.

Ashwell¹⁴² has studied chain stabilization and conductivity enhancement in a range of symmetrical bipyridinium-TCNQ complexes in which the geometry of the cation has been systematically varied. Novel crystal packing is observed with TCNQ molecules forming segregated stacks between which the cations are aligned lengthways. Thus the cation length controls the stoichiometry of the complex. The most significant data have been obtained for (DEPE) $(\text{TCNQ})_4$ (structure 35); the material has very high room temperature conductivity [σ_{rt} 150–2,200 $(\Omega \text{ cm})^{-1}$] and there is a monotonic increase in conductivity on cooling at ambient pressure to 30 mK.

Complexes to TCNQ with *N*-methyl-*N*-ethylmorpholinium (MEM, structure 36) and *N,N*-diethylmorpholinium (DEM, structure 37) cations have been thoroughly studied. $\text{MEM}(\text{TCNQ})_2$ is a room temperature semiconductor [σ_{rt} $10^{-3} (\Omega \text{ cm})^{-1}$] with strongly dimerized stacks: Two phase transitions occur; at 20 K there is a spin Peierls distortion with tetramerization of the TCNQ chains and the material becomes insulating, whereas at 335 K there is an electronic Peierls transition and the high-temperature phase is characterized by uniform TCNQ stacks and metallic conductivity^{143–146}.

$\text{DEM}(\text{TCNQ})_2$ differs structurally from $\text{MEM}(\text{TCNQ})_2$ and offers the unusual chance for studying two inequivalent TCNQ stacks in a single compound. Electron spin resonance¹⁴⁷ and spin susceptibility measurements¹⁴⁸ suggest that on cooling, one of these inequivalent stacks undergoes a spin Peierls transition at 30 K, while the other stack remains in the original high-temperature state. A remarkable range of complexes is formed between DEM and 2,5-dibromo-TCNQ (structure 25); four complexes of stoichiometry 1:1, 3:4, 2:3, and 1:2 have been characterized, and the X-ray structures have been determined for the 1:1 and 1:2 complexes^{149,150}. All four complexes have low conductivity with dimerized acceptor stacks at room temperature but the 1:2 and 2:3 complexes are semiconductors at 450 K^{150,151}.

Applications

A lot of interest in the complexes described here has stemmed from their technological potential. However, as yet these materials have not found extensive application partly because of the frailty of the crystals whereas conducting polymers, such as polyacetylene, are industrially more adaptable. However, the uses of conducting single crystals in microcircuitry of electronic devices have been widely studied. Photoinduced switching between two stable resistance states has been observed in semiconducting films of Cu-TCNQ and Cu-TNAP^{152–154}, and in some TCNQF_4 salts¹⁵⁵. Proposals have been made for organic metals as components of solar cells. Their photoelectric and rectifying behaviour resembles conventional Schottky barrier or MIS (metal-insulator-semiconductor) devices, and research in this area is now concentrated on optimising the nature and distribution of dopants in the organic film to give maximum efficiency^{156,157}. Solid state electrochemical cells based on conducting complexes have been studied since the early TCNQ work¹⁵⁸. Surface modification of conventional electrodes with

TTF-TCNQ has resulted in the first observation of electron transfer from an organic solute to a compacted organic electrode^{159,160}. Halide salts of TTF have potential as lithographic resist materials in the printing industry¹⁶¹.

Our increased understanding of the electrical properties of organic metals may well have implications for the roles of charge carriers in biological systems, as highly conducting porphyrin complexes are well characterized¹⁶². The electron-acceptor properties of TCNQ have found many diverse applications: for example, TCNQ has been used for the study of polar head transitions in some phospholipids¹⁶³, for characterizing electron donor properties of metal oxides used in catalysts¹⁶⁴, and for determining critical micelle concentration of surfactants¹⁶⁵. If superconductivity can be achieved at higher temperatures than those required to date then the technological potential is enormous; more efficient electric motors, very powerful electromagnets and supercomputers that exploit the small heat output of superconducting circuit elements have all been considered¹⁶⁶.

Conclusions

We have highlighted some of the major advances in the field of organic metals. Note that there is overlap between the work covered herein and work on conducting polymers and conducting transition metal complexes, and several theories have developed concurrently in these different areas. So although the problem of a one-dimensional metal has long fascinated solid-state physicists, it was not until the synthesis of TTF-TCNQ, the archetypal organic metal, that the subject began to expand and bear fruit. To a degree unrivalled among other intrinsic conductors, organic metals afford the opportunity for chemical manipulation of the structure and properties of conducting materials. The interdisciplinary synergism within the physical sciences has kept the field at the forefront of research for the past decade. It has become clear that one-dimensional materials require a very precise combination of structural and electronic properties; we have seen repeatedly how minor changes to a donor or acceptor can greatly affect the properties of the complex. The search for high-temperature superconductivity remains a major driving force. In one sense there is a feeling of frustration that the intensive investigation of new donors and acceptors has not produced materials that are significantly more metallic at room temperature than TTF-TCNQ, but many of the complexes, even the insulators, have provided valuable information and helped a more coherent picture to emerge. The work of Bechgaard, Jérôme and others on (TMTSF)₂X salts is directing the impetus towards quasi-two-dimensional solids that will be approached throughout the 1980s with the full resources of organic chemistry, X-ray crystallography, experimental and theoretical physics.

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ARTICLES

Interleukin-3 supports growth of mouse pre-B-cell clones *in vitro*

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Study of the differentiation of immunoglobulin-producing B lymphocytes has been hampered by the inability to maintain homogeneous populations of precursor cells in vitro. We describe here that interleukin-3 supports the growth of freshly isolated fetal liver pre-B cells and the long-term culture of interleukin-3 dependent pre-B-cell clones that can be induced to mature into antibody secreting cells in vitro.

B LYMPHOCYTES arise from multipotential stem cells in the fetal liver, bone marrow and spleen^{1,2}. Several steps occur in their differentiation into immunoglobulin-(Ig) producing cells but these are poorly understood because the subset committed to the B-lymphocyte lineage has not been defined. The major obstacle to the study of the pathway from lymphoid progenitor to pre-B cell and then to mature B cell has been the lack both of techniques for the continuous growth of homogeneous normal cell populations that represent distinct stages of B-cell differentiation and information on the identity or even the existence of growth factors for early B-cell precursors. Some studies report mediators that support short-term replication of mature B cells^{3–5} but, notwithstanding a few reports^{6,7}, long-term culture and exponential growth of B cells *in vitro* has not been achieved. Indeed, it has only recently become possible phenotypically to identify and isolate viable precursor cells belonging to the B-cell lineage^{8,9}.

Here we present cellular, biochemical and molecular evidence for the establishment of factor-dependent mouse pre-B-cell clones in culture and identify interleukin-3 (IL-3) as a growth factor for a population of mouse B-cell precursors.

Factor-dependent Ea3 clones

The Ea3 cell line was derived from the spleen of a *nu/nu* BALB/c mouse. These cells have been grown for over 18 months in medium containing 10% of supernatants from the mouse leukaemic cell line WEHI-3 (WEHI-CM). The Ea3 cells are euploid and do not form tumours in *nu/nu* mice.

Six clones were obtained by cloning Ea3 cells either in soft agar (Ea3.2 and Ea3.11) or by micromanipulation (clones 10, 13, 15 and 17). The cells were collected every 2.5 days and diluted 1:10 in flasks containing WEHI-CM. The growth of both the parent cell line and the Ea3 clones is totally dependent on WEHI-CM. On log phase clones 2, 11, 13, 17 and the parent cell line have a mean generation time of 15–17 h, while in clones 10 and 15 it is 20 h and 19 h, respectively.

WEHI-3 cells produce IL-3¹⁰, haematopoietic cell growth factor (HCGF)¹¹ and burst promoting activity (BPA)¹². These factors have been shown to promote the growth of precursor cells of various haematopoietic lineages^{11–13}. We considered whether they were responsible for the growth of Ea3 cells. As shown in Table 1, homogeneous IL-3 (ref. 14) efficiently

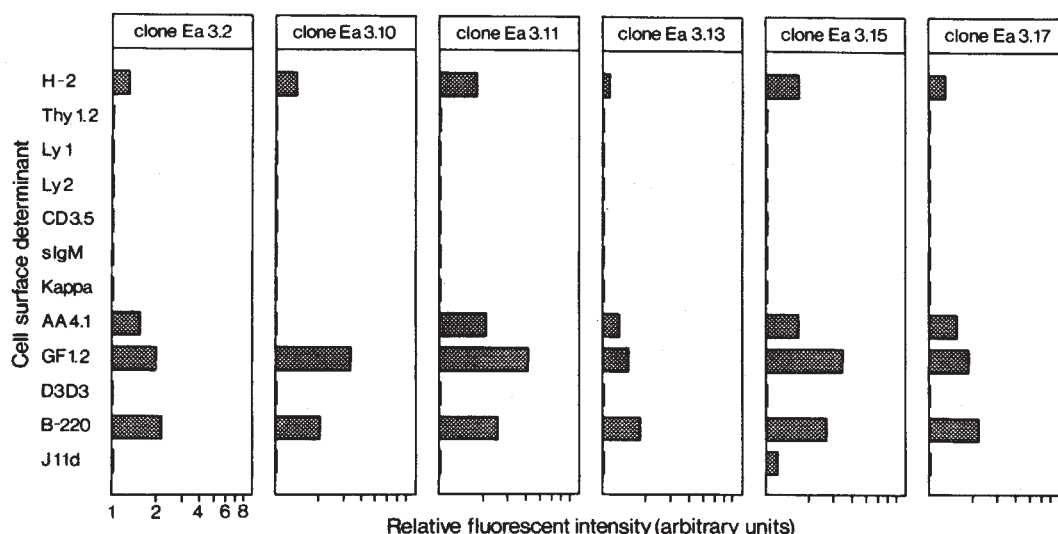


Fig. 1 Quantitation of cell-surface determinants expressed by the Ea3 clones. 2×10^5 viable cells were incubated on ice for 20–25 min with optimum concentrations of rat monoclonal antibodies recognizing various cell-surface determinants. Optimum concentrations were determined using a large panel of transformed cell lines which included pre-B cells, B cells, plasma cells, T cells and a stem cell line as described in detail elsewhere⁸. This analysis provided appropriate positive and negative controls for the FACS assay. Each sample was washed twice and subsequently resuspended in fluorescein isothiocyanate (FITC)–mouse anti-rat immunoglobulin on ice for 20 min. Samples were washed as before, propidium iodide was added to exclude dead cells from analysis and fluorescence was measured using a FACS II (Becton Dickinson) equipped with an argon ion laser. Fluorescence histograms were generated with logarithmic amplification of fluorescence emitted by single viable cells and peak fluorescence channel number was used to calculate antigen density²⁸ using the rat monoclonal antibody D468HLK (anti-rat class I MHC provided by Dr T. J. McKearn) as the negative control since this antibody does not bind to mouse cells. 'Background' histograms generated in this fashion were superimposable on histograms obtained with unstained samples. Almost identical results were obtained in two additional experiments.

Table 1 IL-3 supports the growth of Ea3 cells

	Cytokine	³ H-thymidine uptake of responding cells (c.p.m.)						
		Ea3	Ea3.2	Ea3.10	Ea3.11	Ea3.13	Ea3.15	Ea3.17
Expt 1	Medium	341	286	213	314	297	306	321
	IL-3	12,184	13,128	10,798	13,957	13,194	12,523	14,901
	HCGF	9,723	11,954	6,170	—	10,910	—	—
Expt 2	Medium	228	—	—	—	—	—	—
	IL-3	10,863	—	—	—	—	—	—
	BCGF	231	—	—	—	—	—	—
	BRF	204	—	—	—	—	—	—
	IL-2	242	—	—	—	—	—	—
	IL-1	293	—	—	—	—	—	—

2×10^4 cells either from the parent cell line or from each of the six clones suspended in a final volume of 200 μ l of assay medium were incubated in flat-bottom microplate wells (Nunc Delta AS) in the presence or absence of the following mediators: purified IL-3 (ref. 14) (final dilution of 40 U per ml) interleukin-2 (IL-2) (final concentrations 1–50%) (obtained from phorbol myristate acetate stimulated EL4-16 cells, a cloned line derived from EL-4 thymoma cells kindly made available by Dr Tomas Leanderson), interleukin-1 (IL-1) (final concentrations 1–50%) obtained from the U-937 histiocytic cell line as described elsewhere²⁵, haematopoietic cell growth factor (HCGF) (final dilution 1:250)¹¹, partially purified burst promoting activity (BPA)¹² (final dilution 1:4), B-cell replication factor (BRF) (final concentrations 1–50%) obtained from Con A stimulated A 32-26 T-cell hybrid which produces BRF only¹⁵ or a preparation partially purified of B-cell growth factor (BCGF) (final concentrations 1–50%) (kindly provided by Dr Tomas Leanderson). All these cytokines were checked for their characteristic biological activities and they were very active. Triplicate cultures were incubated at 37 °C for 24 h and 48 h. Cell proliferation was determined by ³H-thymidine uptake (9.25 GBq per well, specific activity 185 MBq, Amersham) during the last 6 h of the culture periods. The results are given as counts per min (c.p.m.) (mean of triplicate cultures where the s.e. was less than 11.3% of the mean).

Methods: The establishment of IL-3 dependent mouse B-cell precursors derived from bone marrow and spleen of both normal and autoimmune mice will be described in detail elsewhere (R.P., J.McK., M.S., J.McC. and G.H., manuscript in preparation). Therefore, we will briefly describe the procedure for the development of the Ea3 cell line here. 2×10^6 spleen cells from *nu/nu* BALB/c mice (7–9 weeks old) (Olac, England) suspended in RPMI-1640 medium (Gibco) supplemented with 10% of heat inactivated fetal calf serum (FCS), gentamycin (50 μ g ml⁻¹, Essex Chemie AG, Lucerne), 2 mM L-glutamine, and IL-3 containing supernatants from the mouse leukaemic cell line WEHI-3 obtained as described previously²⁶ (final concentrations 5–10%) (WEHI-CM), were cultured in 24-well Limbro tissue culture plates (Flow Labs) at 37 °C in a humidified atmosphere of 5% CO₂ in air. After 4–5 days in culture, the non-adherent cells were collected and resuspended in fresh WEHI-CM (5×10^5 cells per ml) and incubated in new plates to deplete adherent cells. This procedure was repeated every 3–4 days, keeping a constant volume of 1.5 ml of WEHI-CM per well. After 7 weeks of culture, Ea3 cells could be transferred into flasks (Nunc Delta) (2.5×10^5 – 5.0×10^5 cells per ml of WEHI-CM). Ea3 cells are dependent on WEHI-CM; in its absence they die within 24–36 h. The Ea3 cells were collected every 2.5 days and diluted 1:10 in fresh WEHI-CM. The clones derived from the Ea3 cell line were obtained either by cloning in soft agar or by micromanipulation. The cloning in soft agar was carried out in 0.2% Bacto agar in Dulbecco's modified Eagle's medium containing 10% FCS overlaid with WEHI-CM in Costar 3524 tissue culture plates. After 7–10 days, single colonies were picked and cultured in Limbro plates containing a final volume of 1 ml of WEHI-CM. Cloning by micromanipulation was performed as described elsewhere²⁷ with the modification that no filler cells were used and the entire procedure was carried out in WEHI-CM. Clones were transferred from 96-well microplates into Limbro plates in a final volume of 1 ml of WEHI-CM. Six Ea3 clones were selected for further study and are reported here. Clones Ea3.2 and Ea3.11 were obtained by cloning the parent line in agar and clones Ea3.10, Ea3.13, Ea3.15 and Ea3.17 were obtained by micromanipulation. The Ea3 clones were maintained in the same conditions as described here for the parent line. Before using Ea3 cells in the assays for proliferative responses described above, the cells were washed three times in balanced salt solution (BSS), resuspended in RPMI-1640 medium supplemented with glutamine and gentamycin (assay medium) and incubated at 37 °C for 5 h. The cells were washed again and resuspended in assay medium at the desired concentrations.

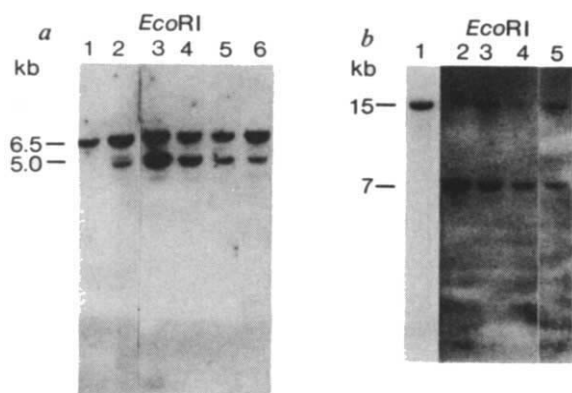


Fig. 2 Southern blot analysis of μ heavy and κ light chain genes. *a*, Hybridization with J_H segments: (lane 1, BALB/c (liver); lane 2, Ea3.2; lane 3, Ea3.11; lane 4, Ea3.13; lane 5, Ea3.17; lane 6, Ea3 parental line). *b*, Hybridization with the C_κ exon: lane 1, BALB/c (liver); lane 2, Ea3 parental line; lane 3, Ea3.11; lane 4, Ea3.13; lane 5, Ea3.17. **Methods:** DNAs from the cell lines were isolated according to Blin and Stafford²⁹ and 10 μ g each was digested with *Bam*HI or with *Eco*RI. DNA fragments were separated on 0.6% agarose gels, transferred to nitrocellulose filters and hybridized with a mixture of the 2 kilobase (kb) *Eco*RI-*Bam*HI and the 1 kb *Bam*HI fragments which contain the four J_H gene segments³⁰ and with a 2.6 kb *Hind*III-*Bam*HI fragment containing the C_κ exon³¹. DNA fragment lengths were calculated from the known molecular weights of λ DNA restriction fragments run in parallel on the same gels. The genes in germ-line configuration are located on the 6.5 kb (*Eco*RI) and the 9 kb and 1 kb *Bam*HI fragments (μ gene) and on the 15 kb *Eco*RI fragment (κ gene).

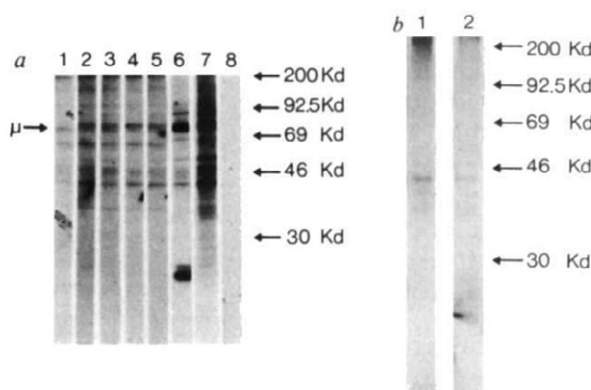


Fig. 3 SDS-polyacrylamide gel electrophoresis (PAGE) of μ heavy chain synthesized by the Ea3 clones. *a*, Lane 1, Ea3.2; lane 2, Ea3.10; lane 3, Ea3.13; lane 4, Ea3.15; lane 5, Ea3.17; lane 6, 204.3.1.6 B-cell lymphoma; lane 7, 70Z/3 cells (anti-immunoglobulin immunoprecipitates); lane 8, 70Z/3 cell lysates (no anti- μ antibody). *b*, Lane 1, Ea3.17 (with control antibody); lane 2, Ea3.17 (without control antibody). The cell line 204.3.1.6 is a $\mu^+ \kappa^+$ B-cell isolate from the Abelson murine leukaemia virus-induced line 204.3.1 (a gift of Dr Naomi Rosenberg)²⁴, and the 70Z/3 cell line is a cytoplasmic μ^+ B-cell lymphoma³². After labelling for 4 h with ³⁵S-methionine (0.5 mCi ml⁻¹, NEN) performed in RPMI-1640 methionine-free medium supplemented with 10% dialysed FCS and L-glutamine, 10⁶ cells were solubilized as described³³. Samples of 100 μ l were precleared with normal rabbit serum and protein A-Sepharose (Pharmacia). Rabbit anti-mouse μ antibody (IgG) or monoclonal mouse IgG2b antibody (control) were added and incubated at room temperature overnight. Incubation of the samples with protein A-Sepharose for 30 min was followed by five Tris-saline, EDTA, 0.5% NP40 washes. Sample buffer³⁴ was added after the last wash to the protein A-Sepharose beads and incubation was at 65 °C for 15 min to release bound material. SDS-PAGE was then performed on 12% Laemmli gels. ¹⁴C-labelled standards (Amersham) were carbonic anhydrase (molecular weight (MW) 30,000), ovalbumin 46,000 MW, bovine serum albumin 69,000 MW, phosphorylase b 92,500 MW and myosin 200,000 MW. The gel was treated in Enlighten (NEN), dried and fluorographed on Kodak XAR-5 film at -70 °C.

supported the growth of the parent line and the six clones derived from it. IL-3 shares most, if not all, of the biochemical and biological properties described for HCGF and BPA¹¹⁻¹⁴, suggesting that all three activities might be carried by the same molecular entity. We found that both HCGF (ref. 11) and BPA (ref. 12) supported proliferation of Ea3 cells (Table 1 and R.P. and N. Iscove, unpublished observations). Consequently IL-3, HCGF and BPA may be the same cytokine but, for brevity, we shall refer to the growth factor of Ea3 cells as IL-3. Neither interleukin-2 (IL-2) nor interleukin-1 (IL-1) showed any growth promoting activity on Ea3 cells (Table 1). Previously reported growth factors for mature B cells, B-cell replication (BFR)³ or B-cell growth factor (BCGF)^{4,5} were also tested. Neither supernatants from the A 32-25 T-cell hybrid which produces BRF only¹⁵ nor partially purified BCGF supported proliferation of Ea3 cells (Table 1). On this evidence the growth factor for Ea3 cells is distinct from replication factors acting on mature B lymphocytes.

We have recently shown that Ea3 cells reversibly adsorb IL-3 but not other unrelated growth factors such as IL-2. Control experiments excluded the possibility that IL-3 activity was lost by degradation or inactivation rather than by adsorption. This suggests that Ea3 cells have specific receptors for IL-3 (ref. 16), which supports growth by interacting with these receptors.

Surface phenotype of Ea3 clones

Ea3 clones stained with Wright-Giemsa are medium to large cells with morphology typical of lymphocytes, having large nuclei and scanty cytoplasm devoid of granules.

The Ea3 clones were characterized by immunofluorescence techniques and fluorescence-activated cell sorter (FACS) analysis using a panel of monoclonal antibodies directed against surface antigens expressed on T cells, plasma cells, B cells, B-cell precursors or cells of myeloid origin. The results are summarized in Fig. 1. All six Ea3 clones lack the T-cell surface markers Thy 1, Lyl 1, Lyl 2 as well as the CD3.5 antigen present on immature T cells (P. Kisielow and J.P.McK., unpublished observations).

Only one of the six clones (clone 15) expressed the Jlld antigen normally found on mature B cells in the spleen and on cells of myeloid lineage in the bone marrow¹⁷. All six Ea3 clones expressed the B220 antigen, a marker for most cells of the B-cell lineage¹⁸, but none of them had the D3D3 antigen which is present on plasma cells and T-cell lymphomas (J.P.McK., J. Slack and J. Davie, unpublished results). All of the clones are positive for the GF1.2 antigen; clones 10, 11 and 15 express higher levels than clones 2, 13 and 17. With the exception of clone 10 they also express the surface antigen AA4.1 (Fig. 1). Monoclonal antibodies GF1.2 and AA4.1 recognize structures present on lymphocytes of B-cell lineage, particularly on B-cell precursors⁸. Finally, none of the Ea3 clones had surface IgM or κ light chains (Fig. 1). In summary, Ea3 clones express the surface antigens B220, GF1.2 and AA4.1, normally present on immature cells of the B-cell lineage. They do not carry surface IgM, κ light chain or the plasma cell antigen recognized by monoclonal antibody D3D3. Thus, the phenotype of Ea3 cells is like that of pre-B cells.

Gene rearrangement

B-cell differentiation from stem cells to immunoglobulin secreting cells is accompanied by rearrangement of immunoglobulin genes. Pre-B cells have been shown to contain a rearranged μ gene formed by joining of the variable region gene segments V_H , D and J (refs 19, 20). Rearrangement of light-chain genes^{21,22} is believed to occur after rearrangement of the μ gene. The configuration of immunoglobulin genes in the parental cell line

Ea3 and in the clones derived from it was studied by Southern blot analysis. DNAs were digested with *Eco*RI and with *Bam*HI, and hybridized with a fragment spanning the four J_H gene segments (Fig. 2a) and with a fragment containing C_κ exon (Fig. 2b). This analysis shows that the Ea3 parent cell line and all the clones contain a germ-line and a rearranged gene for the μ heavy chain as well as for the κ light chain (four clones are shown in Fig. 2a, three in b). The same pattern of gene configuration was found with *Bam*HI DNA digests (that is, a germ-line and a rearranged gene for either the μ chain or the κ chain) (data not shown). Furthermore, the six clones and the parent show the same pattern of bands, indicating that they contain the same rearranged genes. Only quantitative differences in band intensities were found among the seven cell lines. In Fig. 2a, the parental cell line (lane 6) and clone Ea3.2 (lane 2) show a more intensive embryonic band than the band corresponding to the rearranged heavy-chain gene, but the opposite holds for clones Ea3.10, Ea3.11 (lane 3) and Ea3.15. Interestingly, in the latter three clones the band corresponding to the rearranged κ -chain gene is also more intense than its germ-line counterpart. One of these is shown in Fig. 2b (lane 3). It is possible these clones contain multiple copies of the rearranged heavy- and light-chain genes.

Cytoplasmic immunoglobulin

As the Ea3 clones showed immunoglobulin gene rearrangements, we tested whether they also produce μ or κ chains. Immunoprecipitation of the cell lysates with rabbit anti-mouse immunoglobulin antibody showed that all Ea3 clones expressed small amounts of μ chain, but not κ chain in their cytoplasm. The data obtained with clones 2, 10, 13, 15 and 17 are shown in Fig. 3a. The μ and κ chains immunoprecipitated from the B-cell tumour lines 204.3.1.6 and 70Z/3 (used as positive controls) are also shown. The specificity of the μ chain-immunoprecipitation was shown by the lack of bands corresponding to the μ chain in the absence of specific anti-immunoglobulin precipitating antibodies (Fig. 3a, lane 8) or in the presence of an irrelevant antibody (Fig. 3b). Second, the band corresponding to the μ -chain protein was not precipitated in cell lysates from T-helper cell clones.

When tested for secretion of IgM or IgG with the protein A reverse haemolytic plaque assay²³, neither the parent nor any of the clones secreted antibody (Table 2).

IgM secretion

We next investigated whether Ea3 cells could be induced to mature into immunoglobulin-secreting cells *in vitro*. The Ea3 clones were cultured in the presence of irradiated spleen cells and concanavalin A (Con A), on the assumption that Con A would stimulate the irradiated spleen cells to produce all the signals necessary for B-cell maturation, and that in these conditions the only limiting factor would be the capacity of Ea3 cells to differentiate into immunoglobulin-secreting cells. Control cultures were set up either without Con A-treated irradiated spleen cells or without Ea3 cells. The secretion of IgM and IgG was determined by the protein A reverse haemolytic plaque assay²³. The data show that Ea3 clones differentiate into IgM-secreting cells in the presence of Con A-treated irradiated spleen cells (Table 2). IgG-secreting cells were not detected in any of the clones. The fraction of cells secreting IgM after induction varied among the Ea3 clones, clone Ea3.17 having the highest percentage of IgM-secreting cells and Ea3.10 the lowest. No IgM-secreting cells were found in control cultures (Table 2). Immunoprecipitation of extracts from induced Ea3 cells showed that larger amounts of μ chains were produced and the synthesis of the κ chain was induced (data not shown).

The possibility that the IgM detected came from irradiated spleen cells can be excluded on two grounds. First, no IgM-secreting cells were detected in control cultures of Con A-treated, irradiated spleen cells without Ea3 cells (Table 2), and second, no μ or κ chains were precipitated from lysates of these control cultures.

Table 2 Induction of Ea3 clones to differentiate into IgM secreting cells *in vitro*

Cells	Cell assay		IgM PFC per 10 ⁵ viable cells	
	IL-3	Con A stimulated X-ray irradiated spleen cells	Expt 1	Expt 2
Parent cell line	+	—	0	0
Ea3.2	—	+	14,875	12,650
Ea3.10	+	—	0	0
Ea3.11	+	—	23,410	21,745
Ea3.13	—	+	0	0
Ea3.15	+	—	7,480	6,955
Ea3.17	—	+	0	0
None	+	—	8,630	9,340
	—	+	0	0
	+	—	18,195	20,150
	—	+	0	0
	+	—	12,465	15,130
	—	+	0	0
	+	—	28,715	25,480
	—	+	0	0
	+	—	0	0

$6-7 \times 10^5$ Ea3 cells were cultured in the presence of IL-3 containing supernatants (WEHI-CM) or irradiated spleen cells (7×10^6 cells per culture) from normal BALB/c mice in a final volume of 5 ml of culture medium and Con A (Pharmacia, final concentration $2 \mu\text{g ml}^{-1}$) in tissue culture flasks upright at 37 °C for 4–5 days. The cells were collected, washed in BSS and the number of IgM and IgG plaque forming cells (PFC) was determined by the protein A reverse haemolytic plaque assay described by Gronowicz *et al.*²³. Rabbit anti-mouse IgM or anti-mouse IgG used as developing antisera were purchased from Bionetics and used at previously determined optimal dilutions. The results are expressed as IgM PFC per 10⁵ viable cells (mean of duplicate cultures). The irradiated cells from spleens of normal BALB/c mice (8–11 weeks old, Füllinsdorf, Basel) were prepared by standard procedures. Erythrocytes were lysed with ammonium chloride-Tris buffer and the remaining cells were washed three times in BSS, exposed to 2,000 rad of X-ray irradiation, washed again in BSS and resuspended in Iscove's modified Dulbecco's medium supplemented with 10% of heat inactivated FCS, gentamycin ($50 \mu\text{g ml}^{-1}$), and 5×10^{-5} M 2-mercaptoethanol (culture medium).

We conclude therefore that under the influence of maturation signals the Ea3 clones are capable of differentiating into IgM secreting cells *in vitro*.

Growth of pre-B cells

The data obtained with the Ea3 cells suggest IL-3 may act as a growth factor for a population of murine pre-B cells. To study this further we have carried out the following experiments using cells from normal and autoimmune mice.

First, we investigated the proliferative response to homogeneous IL-3 of fetal liver B-cell precursors (from 14th day of gestation) immediately after their isolation. Highly purified fetal liver pre-B cells were obtained by positive selection using the monoclonal antibody AA4.1 which preferentially detects all pre-B cells⁸. This selection technique typically isolated populations which were >95% AA4.1⁺ and 80–85% of which had detectable intracytoplasmic μ chain. AA4⁺ fetal liver pre-B cells were cultured at low cell density (5×10^3 cells per well) with serum-free medium in the presence or absence of homogeneous IL-3 and IL-3 containing supernatants obtained from WEHI-3 (WEHI-CM). Cell proliferation was determined by ³H-thymidine uptake. The results show that homogeneous IL-3 and WEHI-CM efficiently promoted proliferation of these cells (Table 3; expt 1).

The fact that these cells used IL-3 as a growth factor immediately after their isolation, and that they died if cultured in the absence of IL-3, show the dependency of these fetal liver pre-B cells on IL-3 *in vitro* and suggest that this factor dependency may also occur *in vivo*.

Second, we have established additional continuously proliferating pre-B-cell lines from bone marrow and spleen of

Table 3 IL-3 dependent growth of pre-B-cell lines derived from normal and autoimmune mice

Cell line	Strain	Organ	Time in culture	Medium	Proliferative response to cytokines (³ H-thymidine uptake, c.p.m.)				BRF	IFN-γ
					WEHI-CM	IL-3	IL-2	IL-1		
Expt 1										
AA4.1 ⁺	BALB/c	Fetal liver	Fresh	236	13,174	12,638	—	—	—	—
Expt 2										
SJ/B	SJL	Marrow	8 months	417	—	9,365	582	491	407	501
SJ/L	SJL	Spleen	6 months	638	—	8,043	809	724	845	583
L/BE ₁ E ₅	MRL/lpr-lpr	Marrow	10 months	870	—	10,161	765	1,011	904	784
10T4 ⁻	B10T.6R	Marrow	7 months	284	—	10,793	401	275	309	241
Ba/B ₂	BALB/c	Spleen	2.5 months	556	—	12,348	778	812	693	724
NB/W	(NZB/W)F ₁	Marrow	5.5 months	604	—	7,612	750	940	817	682
NZ/S	(NZB/W)F ₁	Spleen	4 months	800	—	12,710	952	862	998	725

Purified AA4.1⁺ fetal liver cells (5×10^3 cells per flat-bottom well) were cultured in 200 μ l of serum-free assay medium (see legend to Table 1) containing homogeneous IL-3 (40 U per ml) or WEHI-CM (final concentration 10%). Cell proliferation was measured by ³H-thymidine uptake (9.25 Gbq per well) during the last 6 h of a 48-h culture period conducted at 37 °C. The proliferative responses of pre-B cell lines described in expt 2 were carried out as detailed in Table 1. The source and concentrations of IL-3, IL-2, IL-1 and BRF used in these experiments were the same as those indicated in the legend to Table 1. Recombinant murine γ -interferon (a gift of Dr P. W. Gray, Genentech and made available to us by Dr Tomas Leanderson) was used at a final concentration of 60 U per ml (this concentration was optimal for inducing the tumour cell line 70Z/3 to express surface IgM). The data are expressed as c.p.m. (mean of triplicate cultures).

Methods: Positive selection of AA4.1⁺ cells was performed as described previously⁸. Briefly, purified AA4.1 antibody was added to Falcon 1001 polystyrene dishes at a concentration of 10 μ g ml⁻¹ in 0.05 M Tris, 0.15 M NaCl pH 9.5 buffer in a total volume of 3–5 ml. Following incubation at room temperature (50–60 min), dishes were rinsed four times with PBS containing 1% FCS and then incubated for 20–30 min with PBS/1% FCS solution to block remaining sites for protein binding. Dishes were then rinsed 4–5 times in BSS. BALB/c fetal liver cells (20×10^6) (obtained at the 14th day of gestation) were layered onto antibody-coated plates in 5 ml of BSS and incubated for 60 min at 4 °C. Cells were redistributed with gentle swirling at 30 min. Non-adherent cells were collected after the 1 h incubation step by decanting the medium and rinsing twice. Following 6–8 additional rinses, the adherent cell fraction was collected by vigorously pipetting 5–7 ml of BSS onto the plates. This was repeated until nearly all cells were detached from the plates. The method for the establishment in culture of IL-3 dependent mouse B-cell precursors from normal and autoimmune mice will be described in detail elsewhere (R.P. *et al.*, manuscript in preparation). Briefly however, bone marrow cells or T-cell depleted (by treatment with anti-Thy 1 antibody and rabbit complement) spleen cells were passed through nylon-wool columns. This procedure enriches for B-cell precursors and decreases the number of precursors of myeloid origin which suppress the growth of mouse B-cell precursors promoted by IL-3 (R.P., unpublished observations). The cells eluted from the nylon-wool columns were washed with BSS and resuspended in WEHI-CM. 2×10^3 cells were incubated in Limbro tissue culture plates in a final vol of 0.5 ml of WEHI-CM at 37 °C for 4–5 days. Every 3–4 days, half of the medium was removed and replaced by fresh WEHI-CM. When the cell cultures were confluent, the cells were distributed in three new Limbro wells suspended in 1 ml of WEHI-CM. After two or three transfers into new wells, the cell lines were transferred into 50-ml tissue culture plastic flasks at a cell concentration of 2.0×10^5 to 4.0×10^5 cells per ml of WEHI-CM. Every 2–3 days the cells are collected and diluted 1:8 in fresh WEHI-CM. The phenotype and biochemical characterization of the pre-B-cell lines will be reported in detail elsewhere (R.P. *et al.*, manuscript in preparation).

various mouse strains with IL-3. Table 3 (expt 2) summarizes the mouse strain, origin and time in culture of several such cell lines. The biochemical characterization of these and other cell lines will be described in detail elsewhere. We have classified these cell lines as pre-B cells according to the following criteria: (1) All have the B-cell lineage antigens B220, AA4.1 and GF1.2, but they do not carry IgM or κ chains on their surface, T-cell markers (Thy 1, Lyt 1, Lyt 2, CD3.5), the myeloid antigen J1d or the D3D3 antigen present on plasma cells. (2) The immunoglobulin genes coding for μ heavy chain are rearranged and the genes coding for κ light chain are in germ-line configuration. (3) These cell lines express messenger RNA for μ heavy chain, but not for κ light chain. (4) Some of them have small amounts of cytoplasmic μ chain.

The results in Table 3 show the growth dependency of these cell lines in IL-3. The cell lines proliferate in response to homogeneous IL-3, but not to other cytokines such as IL-1, IL-2, BRF or γ -interferon. We stress that these experiments were carried out in serum-free medium excluding any putative co-stimulatory activity of serum products. On this evidence, we propose that IL-3 promotes the growth of a population of mouse B-cell precursors.

Conclusions

Four lines of evidence have been presented which permit us to classify the continuously proliferating factor dependent Ea3 clones as pre-B cells. First, Ea3 clones have surface antigens characteristic of the B-cell lineage (B-220, GF1.2 and AA4.1) and they do not express detectable μ or κ chains on their cell membrane. Second, the immunoglobulin genes coding for μ heavy and κ light chains are rearranged in the Ea3 clones. Third, immunoprecipitations of the cell lysates with rabbit anti- μ antibody show that the Ea3 clones express small amounts of μ

chains in their cytoplasm. Fourth, the Ea3 clones can be induced to differentiate into IgM-secreting cells *in vitro*.

Others have reported rearrangement of both J_H gene clusters in Abelson murine leukaemia virus-transformed pre-B cells. These studies suggest that rearrangement of both J_H alleles is an early event in B-cell differentiation²⁴. Our finding demonstrating that the Ea3 clones contain a germ-line and a rearranged J_H gene does not support this view. It seems that dual J_H rearrangement does not necessarily reflect normal events during B-cell differentiation.

The factor required for the growth of Ea3 cells is IL-3; homogeneous preparations of IL-3 support their growth, and they bind purified IL-3 but not other growth factors (such as IL-2). This suggests the presence of specific receptors for IL-3 on their cell membrane¹⁶.

The reproducibility with which mouse B-cell precursor lines can be established in culture with IL-3, and the fact that the growth of all these cell lines is absolutely dependent on IL-3, define IL-3 as a growth factor for a population of mouse B-cell precursors.

We have not been able to establish cell lines of surface Ig⁺ (mature) B lymphocytes with IL-3 nor have we observed any growth promoting activity of IL-3 on purified populations of surface Ig⁺ cells. Thus, IL-3 does not seem to act on mature B lymphocytes. Because growth factors for mature B cells (BRF/BCGF) described previously do not promote proliferation of Ea3 cells (Table 1) or of other pre-B cell lines derived from normal and autoimmune mice (Table 3), distinct growth factors act on B-cell precursors and on mature B lymphocytes. It is possible that during differentiation, IL-3 dependent pre-B cells lose the capacity to express receptors for IL-3 as they mature. The expression of surface immunoglobulin might coincide with the loss of the capacity to bind IL-3 and the acquisition of the

ability to express receptors for BRF/BCGF upon interaction with antigen and Ia-restricted T-helper cells.

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Duplication within the haptoglobin Hp^2 gene

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DNA sequencing shows that the intragenic duplication within the human haptoglobin Hp^2 allele was formed by a non-homologous, probably random, crossing-over within different introns of two Hp^1 genes, probably in an Hp^{1F}/Hp^{1S} heterozygote.

It has been demonstrated by one of us and his collaborators^{1–6} that human haptoglobin, a haemoglobin-binding serum protein, has inherited variations as a consequence of the presence in most populations of three common autosomal alleles, Hp^{1F} , Hp^{1S} and Hp^2 . The Hp^{1F} and Hp^{1S} alleles were shown to code for polypeptides of equal length, $hp1F\alpha$ and $hp1S\alpha$, differing in charged amino acids which make them migrate as fast and slow bands during electrophoresis in acidic starch gels. The Hp^2 allele differed in coding for a polypeptide chain ($hp2\alpha$) almost twice as large as the $hp1F\alpha$ and $hp1S\alpha$ chains. As $hp2\alpha$ polypeptide contained peptides related to both the $hp1F\alpha$ and $hp1S\alpha$ polypeptides, Smithies, Connell and Dixon⁶ postulated that the Hp^2 allele contained a partial gene duplication (an intragenic duplication) formed by a rare non-homologous crossing-over event which permitted fusion of the Hp^{1F} and Hp^{1S} genes in a heterozygote. Because the intragenic duplication permitted the reading frame to be preserved in the larger $hp2\alpha$ polypeptide, the haptoglobin duplication suggested mechanisms for building new genes for new proteins from parts of old genes for other proteins. The work emphasized the distinction between unique and unpredictable non-homologous types of events, such as that postulated in forming the Hp^2 intragenic duplication, and subsequent predictable events expected from unequal but homologous crossing-over involving the duplication in the Hp^2 gene. Among the predicted recombinants were four types of Hp^2 gene, designated Hp^{2FS} , Hp^{2SF} , Hp^{2FF} and Hp^{2SS} depending on whether the genes had their duplicated regions like Hp^{1F} or Hp^{1S} . Subsequent studies⁷ showed the existence of electrophoretic variants of the $hp2\alpha$ chain having the predicted properties.

Within the past year, vander Straten *et al.*⁸, Yang *et al.*⁹ and Raugei *et al.*¹⁰ have cloned and sequenced cDNA corresponding to the Hp^2 allele; in each case it is of the Hp^{2FS} variety. The nucleotide sequences reported for the cDNA also show that the

β polypeptide chain of haptoglobin must be translated co-linearly with the α -chain, agreeing with the suggestion of Barnett *et al.*¹¹ that haptoglobin might be synthesized as a single polypeptide chain followed by proteolytic cleavage. We have used this cloned haptoglobin cDNA to isolate genomic DNA sequences corresponding to the three common haptoglobin alleles, Hp^{1F} , Hp^{1S} and Hp^2 , together with DNA corresponding to the additional haptoglobin gene or pseudogene reported by Raugei *et al.*¹⁰. Here we describe these isolations and present the nucleotide sequence of 5 kilobase pairs (kbp) of the common Hp^2 allele (Hp^{2FS}) and of parts of the Hp^{1F} and Hp^{1S} alleles. These nucleotide sequences verify and enrich the earlier inferences from studies of the haptoglobin proteins.

Haptoglobin genes

We mapped the haptoglobin genes in the DNA of several individuals with known haptoglobin phenotypes using cDNA probes: an $hp2\alpha$ probe covering the region coding for the $hp2\alpha$ polypeptide and its leader, and an $hp\beta$ probe covering the 5' two-thirds of the region coding for the $hp\beta$ polypeptide chain (see Fig. 1 legend for details). Figure 1a–c presents Southern blots¹² hybridized to these probes, while Fig. 1d presents the maps deduced from these blots combined with data from cloned fragments. Data obtained with the $hp2\alpha$ probe (Fig. 1a, b) indicate that the length of the duplicated part of the Hp^2 allele is 1.7 kbp.

Hp^{1F} and Hp^{1S} can be distinguished after digestion with *Xba*I, as Hp^{1F} gives a 4.6-kbp fragment while Hp^{1S} gives a 4.0-kbp fragment (see Figs 1b, 2). However, the Hp^2 allele also gives a 4.6-kbp fragment with *Xba*I, so this enzyme will not distinguish Hp^{1F} from Hp^2 .

The $hp\beta$ probe hybridizes to the same fragments as does the $hp2\alpha$ probe in *Eco*RI and *Hind*III digests of genomic DNA, indicating that the coding regions for the α - and β -chains of

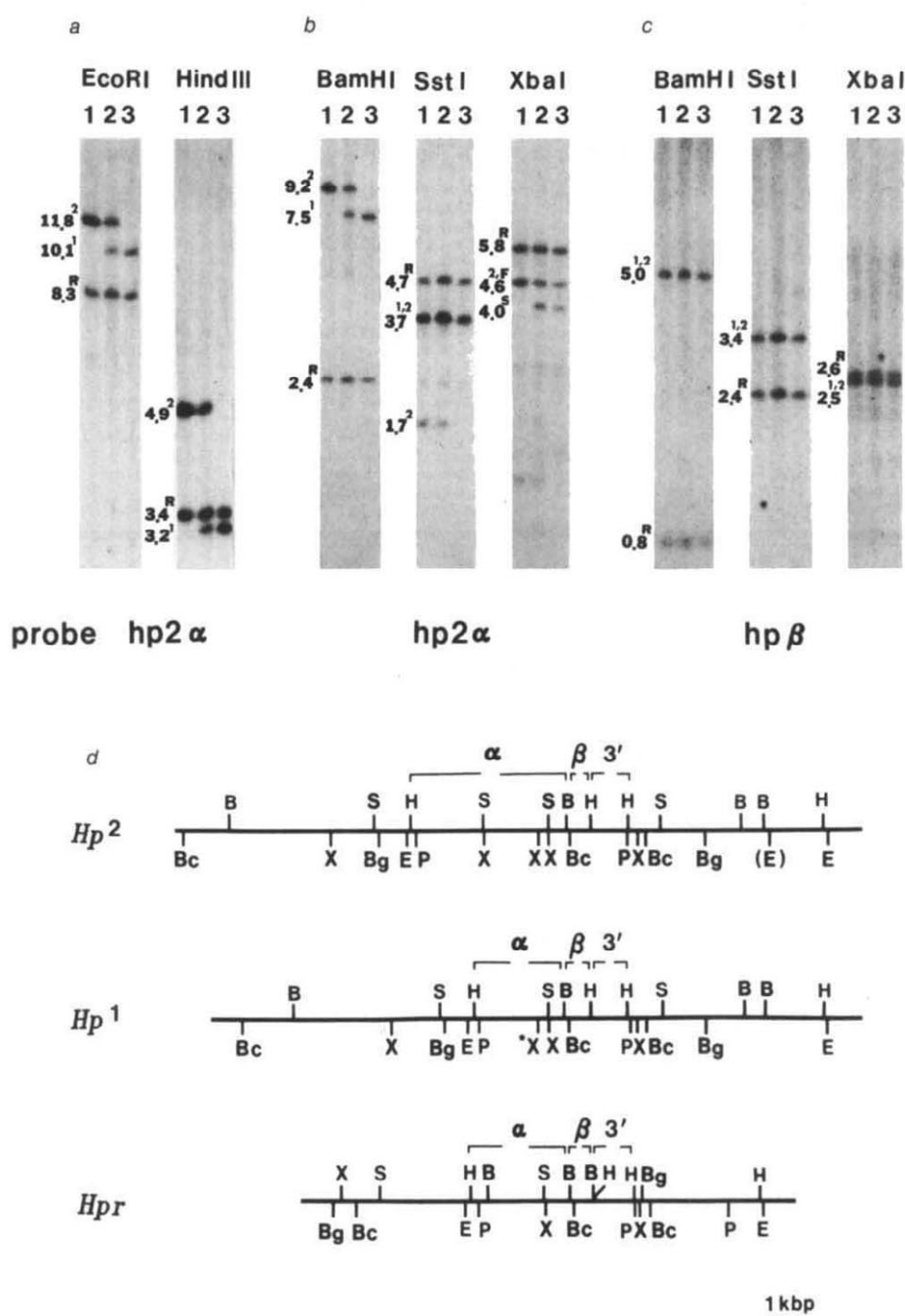


Fig. 1 *a-c*, Southern blot analysis of genomic DNA from individuals with haptoglobin genotypes Hp^2/Hp^2 , Hp^2/Hp^{1S} and Hp^{1F}/Hp^{1S} (lanes 1, 2, 3). *a*, *EcoRI* digests and *HindIII* digests hybridized to the $hp2\alpha$ probe. (The $hp\beta$ probe hybridized to the same bands.) *b*, *BamHI*, *SstI* and *XbaI* digests hybridized to the $hp2\alpha$ probe. *c*, The filter from *b* rehybridized to the $hp\beta$ probe after removing the $hp2\alpha$ probe by incubating the filter in distilled water at 90 °C for 10 min. Bands derived from the Hp^2 , Hp^1 genes and the haptoglobin-related (Hpr) sequences are superscripted 2, 1 and R respectively. F superscripts, bands from Hp^{1F} ; S, Hp^{1S} . *d*, Restriction enzyme maps of the Hp^2 and Hp^1 alleles and the Hpr sequences. The maps were generated from blots of digests of genomic DNA together with results from isolated clones. The regions hybridizing to the $hp2\alpha$ and $hp\beta$ probes and a 3' probe are shown by brackets labelled α , β and 3' respectively. (The 3' probe covers the last 90 coding nucleotides of the haptoglobin β -chain and 200 nucleotides from the 3'-untranslated region.) A polymorphic *EcoRI* site (see text) is indicated by (E). An *XbaI* site present in Hp^{1F} and absent in Hp^{1S} is indicated by an asterisk. Abbreviations for the restriction sites are: B, *BamHI*; Bc, *BclI*; Bg, *BglII*; E, *EcoRI*; H, *HindIII*; P, *PstI*; S, *SstI*; X, *XbaI*.

Methods: *a-c*, DNA was prepared from white blood cells from the three individuals and digested to completion with the enzymes indicated. The digests (3 μ g) were electrophoresed in 0.8% agarose gels, transferred to nitrocellulose filters and hybridized to the radioactive probes indicated. The $hp2\alpha$ probe is a 500-bp *PstI/BamHI* fragment covering the region encoding the $hp2\alpha$ polypeptide and its leader; the $hp\beta$ probe is a 650-bp *BamHI/HindIII* fragment covering the 5' two-thirds of the $hp\beta$ polypeptide coding region. Filters were washed at 68 °C for 30 min twice with 3 $\times$ SSC containing 0.5% SDS, and once with 0.3 $\times$ SSC, 0.1% SDS.

haptoglobin are close together in the genome. However, *SstI* and *XbaI* digests have different patterns with the two probes (see Fig. 1c), even though there are no *SstI* or *XbaI* sites in the $hp2$ cDNA sequence. Consequently, there must be an intervening sequence containing *SstI* and *XbaI* sites between the α -chain and β -chain hybridizing regions.

An additional band (labelled R in Fig. 1a-c) hybridizes to both the $hp2\alpha$ and $hp\beta$ probes, does not vary with the Hp genotype and is seen in all digests. This haptoglobin-related sequence was previously noted by Raugei *et al.*¹⁰. We shall refer to the corresponding locus as *Hpr* (for haptoglobin-related).

Figure 1 shows that the three common Hp alleles are readily distinguished by Southern blots with the $hp2\alpha$ probe. The Hp locus thus provides a convenient restriction fragment length polymorphism (RFLP) for use in prenatal or other linkage studies at the DNA level.

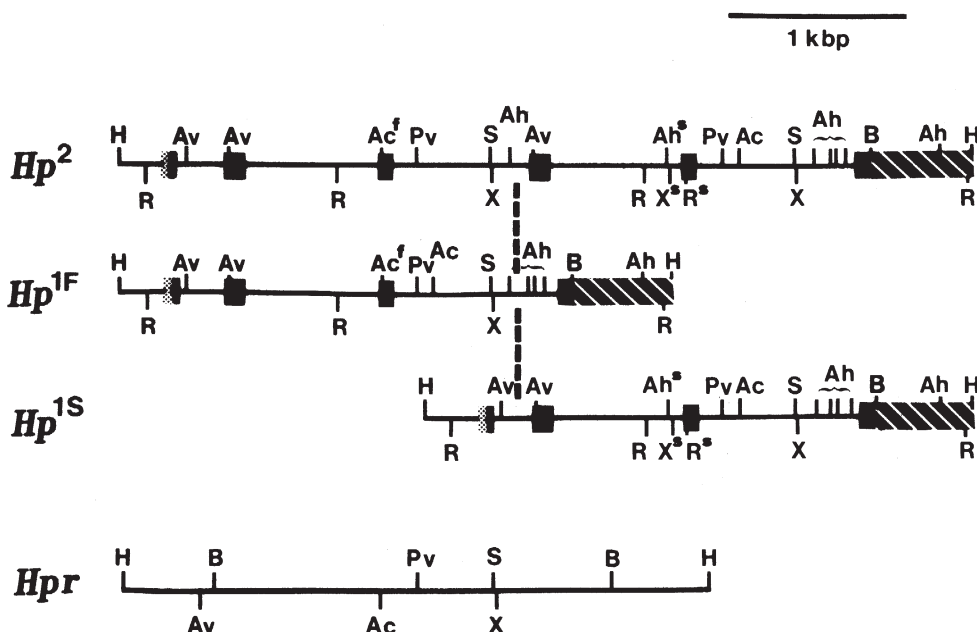
Close to the Hp locus there is a polymorphic *EcoRI* restriction enzyme site (previously noted by Raugei *et al.*¹⁰) which provides an additional RFLP in the same general vicinity as the Hp locus.

We have mapped this polymorphic *EcoRI* site to the position indicated (E) in Fig. 1d, approximately 4 kbp 3' to the Hp gene. So far the polymorphic site has been found only in association with Hp^2 , judging from two examples shown by Raugei *et al.*¹⁰ and one individual of Italian descent that we have observed. The presence of this *EcoRI* site in association with the Hp^2 allele changes the size of the *EcoRI* fragment hybridizing to the $hp2\alpha$ probe from 11.8 kbp (see Fig. 1a) to 10.1 kbp, which is the same size as the *EcoRI* fragment derived from Hp^1 genes. This makes *EcoRI* an unsuitable enzyme for Hp genotyping.

Isolation and mapping

The fragments in complete *EcoRI* digests of DNA from individuals with the genotypes Hp^2/Hp^{1S} and Hp^{1F}/Hp^{1S} were ligated into DNA from the bacteriophage vector Charon 32 (ref. 13). The ligated DNAs were assembled into infective bacteriophages using the *in vitro* packaging procedure of Hohn¹⁴ and screened for hybridizing plaques using a mixture of $hp2\alpha$ and $hp\beta$ probes. Phages were obtained containing *EcoRI* frag-

Fig. 2 Detailed maps of the *Hind*III fragments from the Hp^2 , Hp^{1F} and Hp^{1S} genes and from the *Hpr* sequences. Coding regions are indicated by a stippled box for the leader sequence, by solid black boxes for the α -chain, and by striped boxes for the β -chain. The broken vertical line shows the location of the breakage and reunion that generated Hp^2 . Ac, *AccI*; Ah, *AhaIII*; Av, *AvaI*; R, *RsaI*; Pv, *PvuII*. Other enzyme sites are as in Fig. 1.



ments of the three sizes expected for Hp^1 , Hp^2 and *Hpr* (see Fig. 1a): 10.1, 11.8 and 8.3 kbp.

The Hp^1 -containing phages could be divided into two types corresponding to Hp^{1F} and Hp^{1S} on the basis of *XbaI* and *AccI* digests. We show below that the Hp^2 allele obtained here was of the Hp^{2FS} variety, as were the three independent cDNA clones described previously⁸⁻¹⁰. Clearly, Hp^{2FS} is the common Hp^2 allele, so we refer to it below as Hp^2 without the FS designation.

*Hind*III fragments corresponding to Hp^{1F} , Hp^{1S} , Hp^2 and *Hpr* were subcloned into p010Δori (ref. 15) and their detailed restriction enzyme maps were prepared. Figure 2 presents these maps and shows the locations of coding and noncoding regions identified below from nucleotide sequences.

The Hp^2 allele

Figure 3 presents the nucleotide sequence of ~5 kbp of DNA from the Hp^2 allele determined by the Maxam-Gilbert procedure¹⁶. All parts of the sequence have been determined from at least two different restriction sites, and about 80% of the sequence has been determined on both strands of the DNA. In all but a few positions our gene sequence and the published cDNA sequences agree in the coding regions. The exceptions are that (1) amino acid position 52 of the hp2α chain is aspartic acid (GAT) in our sequence—this differs from the AAT (asparagine) of Yang *et al.*⁹, but is identical to the sequences of vander Straten *et al.*⁸ and Rauegi *et al.*¹⁰; (2) the codons for leucine at positions 2 and 6 in our β-chain gene sequence are both CTG, whereas the cDNA sequences show CTG and TTG (ref. 8), CTA and CTT (ref. 9) and CTG and CTT (ref. 10). We find that the Hp^{1S} gene also has CTG for both these leucine residues (data not shown). The source of these differences cannot be determined from the existing data.

In presenting the sequence we have aligned the duplicate portions of the Hp^2 allele one below the other to show clearly the extent and end points of the duplication. The coding regions are also indicated. We find six intervening sequences within the Hp^2 gene, which break the coding region into seven exons. As the Hp^2 gene has not been completely sequenced, we cannot be certain that there are only seven exons. However, electron microscopy and heteroduplexes between the Hp^2 genomic DNA and the hp2 cDNA (Meg Ulber, personal communication) agrees with this number and shows no introns in the hpβ-coding region. There are two fewer exons in Hp^1 than in Hp^2 genes.

The end points of the intragenic 1,725-bp duplicated region within the Hp^2 allele are easily seen from the alignments in Fig. 3; they lie within two different introns. Sequence data show why

the duplication did not disturb the reading frame of the hp2α polypeptide. Although the duplicate segment starts and ends in different introns, and introns can be in any of three reading frames, the RNA splicing donor site of the fourth exon of Hp^1 genes is in the same reading frame as the acceptor site of the third exon of Hp^1 genes. Consequently, the reading frame is not disturbed by the duplication which causes the fourth exon of an Hp^1 gene to be separated by an intron from the third exon of another Hp^1 gene.

The protein sequence data indicate that the duplicated region corresponds to a nearly perfect repeat of 59 amino acid residues¹⁷. The DNA sequence data confirm this. The sequenced duplicate DNA segments do, however, differ slightly from each other in coding for Asp-Lys and Asn-Glu at the equivalent positions 52, 53 and 111, 112. This shows that the present Hp^2 allele is of the Hp^{2FS} variety.

Note that the first halves (790 bp) of the two copies of the duplicated region are identical, whereas the second halves (935 bp) differ at 37 positions (27 transitions, 8 transversions and 2 length differences), with all but four of the differences occurring in noncoding regions. Possibly the first half of the duplicated region has been involved in a recent gene conversion event.

As we have seen, the duplicate parts of the Hp^2 allele differ from each other at 37 positions out of their 1,725 nucleotides (~2%). Two scenarios, not mutually exclusive, could account for these differences: either the Hp^2 allele was formed by duplication of part of an Hp^1 allele followed by divergence of the intragenic duplicates, or the Hp^2 allele was formed by an event involving the illegitimate joining of two already-diverged Hp^{1F} and Hp^{1S} alleles in a heterozygote. The two scenarios can be distinguished (in the absence of too many subsequent mutations or of extensive recombination) by comparing the 5' and 3' copies of the duplicated region in Hp^2 with the equivalent regions in Hp^{1F} and Hp^{1S} . Scenario one predicts that the 5' and 3' copies of the duplicated region will be more similar to each other than they are respectively to Hp^{1F} and Hp^{1S} . Scenario two predicts that the 5' duplicated region will be more similar to Hp^{1F} than to the 3' duplicated region, which in turn should be similar to Hp^{1S} . Inspection of the detailed restriction maps of the three alleles shows that four pairs of restriction sites (marked appropriately with f and s in Fig. 2) are distributed in Hp^2 , Hp^{1F} and Hp^{1S} in accordance with scenario two, but not with scenario one. However, partial sequencing of an Hp^{1F} allele and an Hp^{1S} allele (data not shown) indicates that the true situation is probably more complex, because the sequences show signs of

Fig. 3 The nucleotide sequence of a *Hind*III fragment from the *Hp*² gene. The sequence is presented so that the duplicate regions (nucleotides 480–2,203 and 2,204–3,918) are aligned, with inserted gaps (*) where necessary. Bases identical in the duplicate regions are tied by vertical lines. Numbers on the left refer to nucleotides. Numbers in the body of the figure refer to the amino acid sequence deduced for the α (1–142) and β (1–214) chains. Differences in the amino acid sequences encoded by the *Hp*^{1F} and *Hp*^{1S} genes are marked with overlines and underlines. Exons are indicated. Because the first two residues of the leader sequences (Met-Ser) are not encoded within the sequenced region, we have assumed that the first sequenced exon is exon 2.

parental alleles are inaccessible, we have sequenced the regions in the modern *Hp*^{1F} and *Hp*^{1S} genes which surround the putative breakpoints, and have compared them with the sequence of the region surrounding the middle joining point of the duplicated segments in *Hp*². This comparison (Fig. 4) approximates the situation existing at the time of formation of the *Hp*² gene.



Fig. 4 Details of the crossing-over that formed the Hp^2 allele. Bases identical to Hp^2 are tied by vertical lines. The box shows the region within which the crossing-over occurred.

The comparison shows no obvious similarity between the relevant regions of the Hp^{1F} and Hp^{1S} sequences, although the data localize the point of joining of Hp^{1F} -derived to Hp^{1S} -derived sequences to within a region of a few base pairs (boxed in Fig. 4). However, as the Hp^2 sequence around the joining point is not exactly identical to the equivalent sequence of either of the two Hp^1 genes we have currently sequenced, the joining point cannot be unequivocally identified. Close to the joining point a G-C dinucleotide is common to all three sequences shown in Fig. 4. Except for this, we have found no evidence for local homology between the two Hp^1 sequences in the region where the breakage and reunion occurred in forming the duplication. We thus conclude that the formation of the Hp^2 allele was the result of a breakage and reunion event at non-homologous positions within the fourth and second introns of two Hp^1 genes.

There are a few bases in the Hp^2 sequence that do not appear to be derived from either of the Hp^1 genes; they are within the boxed region in Fig. 4. The origin of these bases of uncertain ancestry remains to be determined. Possibly they resulted from the breakage and reunion event itself; possibly the Hp^{1F} and Hp^{1S} alleles we have sequenced differ from the true ancestors of the Hp^2 gene.

We looked for any unusual features in the sequences which might account for the DNA breakage and reunion at these particular places. We found nothing obvious although ~300 bp upstream of the breakpoint in Hp^{1S} there is a stretch of simple sequence DNA consisting of 33 pairs of alternating purine and pyrimidine nucleotides (almost purely T-G). Such stretches of

(T-G)_n can form Z-DNA¹⁸ and a similar stretch of simple sequence DNA was postulated to be a hotspot for recombination in the human fetal globin genes¹⁹. The region in which the breakpoint occurred in Hp^{1F} is somewhat A+T-rich (~68%), which may have made the region more susceptible to breakage. On the other hand, the breakpoint in Hp^{1S} lies in a slightly G+C-rich region (~54%). Taken together, these data suggest that the breakpoints involved in forming the Hp^2 intragenic duplication were probably not predisposed by local nucleotide sequences.

Conclusion

We have established by nucleotide sequencing that the duplication within the haptoglobin Hp^2 allele was formed by a non-homologous and probably random cross-over within two different introns of two Hp^1 genes, probably in an Hp^{1F}/Hp^{1S} heterozygote. The history deduced from our present studies of the nucleotide sequences of the Hp alleles agrees with the history deduced by Smithies, Connell and Dixon⁶ from studies of the haptoglobin proteins.

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LETTERS TO NATURE

Magnetospheric flux erosion events are flux transfer events

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Recent results have shown that magnetic reconnection at the Earth's dayside magnetopause manifests itself most frequently as an impulsive process. Haerendel *et al.*¹, using data from the HEOS 2 spacecraft, were the first to notice short time scale magnetic field perturbations inside the magnetopause, which they interpreted as flux erosion events resulting from transient reconnection in the polar cusp regions. Using higher time resolution data from the ISEE 1 and 2 satellite pair, Russell and Elphic² subsequently discovered certain characteristic magnetic

field signatures in the magnetosheath, which they termed flux transfer events (FTEs). They also interpreted these events in terms of transient, localized reconnection. It has often been suggested³⁻⁷ that Haerendel *et al.*'s flux erosion events and Russell and Elphic's FTEs have a close physical connection, but proof of this has so far been lacking. Here we show that flux erosion events and flux transfer events are indeed the same physical phenomenon.

Over 20 years ago, Dungey⁸ suggested a model of the Earth's magnetosphere in which he proposed magnetic field reconnection as the major process coupling the magnetosphere to the solar wind and driving magnetospheric convection. Considerable indirect support for this model has subsequently been derived from studies showing the strong response of the magnetosphere to the vector direction of the solar wind magnetic field (for example, refs 9, 10). More recently, *in situ* satellite measurements have provided convincing experimental evidence for the occurrence of reconnection at the Earth's dayside magnetopause, that is, the boundary between the magnetosphere and

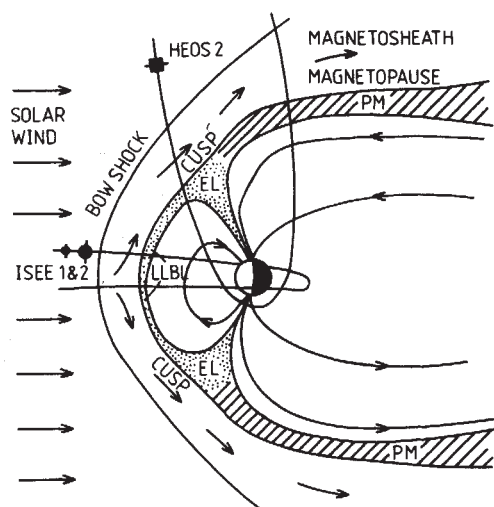


Fig. 1 Sketch in the noon-midnight meridian plane showing a typical orbit of the ISEE 1 and 2 spacecraft and of the HEOS 2 spacecraft in relation to the Earth's magnetosphere. LLBL, EL and PM refer to boundary layer regions of magnetosheath-like plasma inside the magnetopause: they are, respectively, the low latitude boundary layer, entry layer and plasma mantle. HEOS 2 has obtained data from all three boundary layer regions.

the external shocked solar wind (magnetosheath)¹¹⁻¹³. Most frequently, reconnection manifests itself as a process occurring on short space and time scales. The signature of transient reconnection was first recognized by Haerendel *et al.*¹; using data from the HEOS 2 spacecraft (see Fig. 1), they found brief periods of large-amplitude magnetic field perturbations in the low-latitude boundary layer, that is, the layer of magnetosheath-like plasma inside the dayside magnetopause. Russell and Elphic² subsequently discovered certain characteristic magnetic signatures in the magnetosheath, using higher time resolution data from the ISEE 1 and 2 spacecraft (Fig. 1). They interpreted these signatures, called flux transfer events, in terms of open magnetic flux tubes formed as a result of localized and transient reconnection. Further studies of ISEE 1 and 2 plasma and magnetic field data have provided confirmatory evidence for this interpretation and corresponding FTE signatures inside the magnetopause have also been found (see refs 3 and 4 and references therein).

Here, we have re-examined the HEOS 2 data set using the same analysis techniques that were applied to the later ISEE data, and we show that flux erosion events and FTEs are indeed the same physical phenomenon. The easiest way to recognize FTE magnetic signatures is to convert the magnetic field data into a coordinate system referenced to the local magnetopause surface. The use of this so-called boundary normal (LMN) coordinate system, introduced by Russell and Elphic², was partially responsible for the first detailed interpretation of FTEs in terms of transient reconnection events. In this system, the L and M components point roughly northward and westward (dawnward) along the local magnetopause surface, respectively, while the N component points along the outward normal to the boundary. The classic magnetic signature of a FTE consists of a bipolar perturbation in the normal field component B_N coupled with a deflection of the field tangential to the magnetopause (B_L and B_M) and an increase in the total field strength B compared with ambient values.

To determine the relationship between the FTEs of Russell and Elphic² and the flux erosion events described by Haerendel *et al.*¹, some of the HEOS 2 magnetic field data were plotted in the boundary normal coordinate system. Figure 2 shows an example of a magnetopause encounter¹ which occurred at a spacecraft location just duskward of noon and at a geocentric solar magnetospheric (GSM) latitude of 64°. The four panels

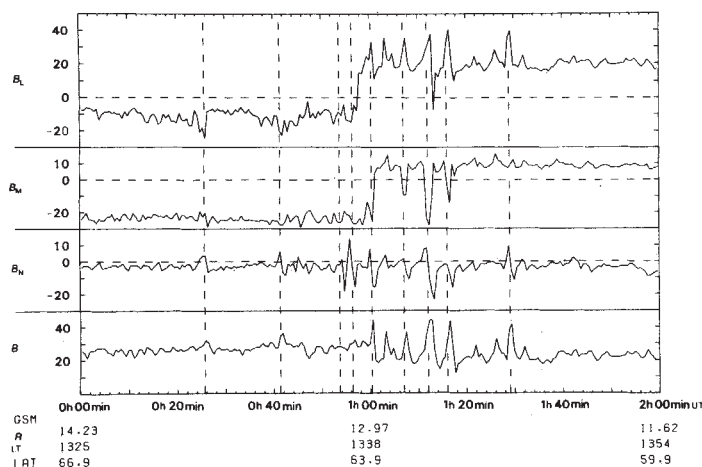


Fig. 2 A 2-h interval of HEOS 2 magnetic field data obtained at the maximum resolution of one data point every 32 s, during the inbound pass of the magnetopause region on 1 December 1973 (day 335). The four panels show the L, M and N components of the magnetic field (see text) and the total field strength B , in nT. The spacecraft location in GSM coordinates is given at the bottom in terms of the radial distance (R) in R_E , the local time (LT) in hours and minutes, and the northerly latitude (LAT). The broken vertical lines indicate the times at which clear FTE signatures (see text) occur.

show respectively the L, M and N components of the magnetic field and the total field strength B , at the full time resolution of one data point every 32 s. The direction of the magnetopause normal, N, was determined from minimum variance analysis¹⁴ of the boundary crossing at ~ 0.58 UT. Its GSM components were determined as (0.649, -0.081, 0.757), which is at an angle of $\sim 11^\circ$ to the Formisano *et al.*¹⁵ model normal at the spacecraft location. At the start of the interval shown, the spacecraft was situated in the magnetosheath, as shown by the southerly and duskward-directed field (that is, B_L and B_M are both negative) and by the plasma data (see ref. 1). The magnetopause, identified from the change in magnetic field orientation, is crossed at ~ 0.58 UT, after which the magnetic field assumes its magnetospheric orientation, that is, northward and dawnward (B_L and B_M both positive). The broken vertical lines in Fig. 2 show instances of magnetic field signatures which we have identified as FTEs. FTEs displaying the classic magnetic signatures described above occur at 0.26, 0.42 and 0.56 UT in the magnetosheath, and at 1.00, 1.12 and 1.29 UT in the magnetopause and magnetosphere regions. If the deflections in the tangential field components (mostly in B_L) and the field strength increases are aligned, it is easy to see that the bipolar B_N signatures are in the positive-negative sense. Three further FTEs also occur at 0.54 UT in the magnetosheath and at 1.07 and 1.16 UT in the magnetosphere. Their signatures in B_N are not strictly bipolar, therefore we class them as irregular according to the scheme of Rijnbeek *et al.*¹⁶. Seemingly related perturbations on a smaller scale also occur at 0.46, 1.03, 1.22, 1.26 and 1.32 UT. The large field perturbations inside the magnetosphere (for example, 1.07, 1.12 and 1.16 UT) are in fact the features described by Haerendel *et al.*¹ as flux erosion events. The presentation of the data in the boundary normal coordinate system provides clear evidence that flux erosion events and FTEs are the same physical phenomenon.

Haerendel *et al.*¹ and Haerendel and Paschmann⁷ suggested that the flux erosion events shown in Fig. 2 result from transient reconnection in the northern polar cusp region, as sketched in Fig. 3a (see also Fig. 14 of ref. 1). Figure 3a shows that as the magnetospheric field orientation (northward and dawnward in the present case) indicates that HEOS 2 was located south of the northern cusp, we should be observing open flux tubes connected to the Earth's Southern Hemisphere. The northward component of the ambient magnetosheath plasma flow opposes

the southward pulling of the southerly connected flux tube in Fig. 3a due to the release of magnetic tension. Hence, Haerendel *et al.*¹ suggested that the increases in field strength during the events in Fig. 2 result from conversion of plasma flow energy into magnetic field energy, through extension of the field lines¹⁷. Also, note that the combined action of the ambient magnetosheath plasma flow and the magnetic tension release will tilt the magnetospheric field lines inside the open flux tubes towards dusk (eastward). Such duskward field tilts are indeed observed inside the magnetosphere for the FTEs at 1.00, 1.07, 1.12 and 1.6 UT. However, a difficulty arises from the interpretation of Haerendel *et al.*¹. In Russell and Elphic's² interpretation of a FTE, the bipolar B_N signature results from the draping of the external unreconnected field lines around the flux tube. Hence, the sense of the B_N signature is determined by the motion of the FTE flux tube across the spacecraft^{3,18}. In Fig. 3a, the FTE flux tube connected to the Southern Hemisphere is carried duskward both by the ambient magnetosheath plasma flow and the release of magnetic tension. The external unreconnected field lines in the magnetosheath and in the magnetosphere will drape over and underneath the FTE flux tube, respectively. The underlying draped magnetospheric field lines will first tilt in the negative N direction and then tilt towards the positive N direction as the flux tube travels with a duskward velocity component across the HEOS 2 location. In other words, we should observe reverse polarity (negative-positive) B_N signatures inside the magnetosphere. Such reverse FTE¹⁹ signatures are clearly not observed in Fig. 2. The B_N signature in the magnetosheath caused by the external magnetosheath field lines draping over the southerly connected tube in Fig. 3a is more difficult to predict. It seems likely, however, that a net northward velocity component of the flux tube across the HEOS 2 location should result in a positive-negative B_N signature (as observed in Fig. 2), whereas a net southward component should produce reverse (negative-positive) B_N signatures (see ref. 18 for a more detailed discussion of this point). Although the difficulty of seeing positive-negative B_N signatures in the magnetosphere in Fig. 2 is not irreconcilable (discussed below) we shall now examine another possibility—that the events in Fig. 2 are open flux tubes connected to the Northern Hemisphere and result from impulsive reconnection equatorward of the HEOS 2 spacecraft, rather than in the northern cusp region.

Figure 3b again shows a view of the frontside magnetopause, this time with reconnection initiated near the Equator. In this situation, northerly and southerly connected open tubes will move northward and southward away from the initial reconnection site, respectively, under the action of both the magnetosheath plasma flow and the magnetic tension release. Therefore, at northerly latitudes such as in Fig. 2, we expect to observe northerly connected flux tubes. If we assume again that the sense of the B_N signature is determined by the draping of external field lines, this picture immediately explains the observed positive-negative B_N signatures observed in the magnetosheath and in the magnetosphere; see Fig. 3b (refs 3, 18). The northward motion of the northerly connected flux tube in Fig. 3b across the spacecraft location will cause the external draped field lines in the magnetosheath and in the magnetosphere first to tilt in the positive N direction and then to tilt in the negative N direction. Meanwhile, the observed duskward field tilts during the post-noon magnetospheric FTEs in Fig. 2 should result from the effects of the magnetic tension rather than the magnetosheath flow dominating the direction of east-west motion of the reconnected flux tubes inside the magnetosphere (see ref. 20 for discussion). In Fig. 3b, for example, the field tension will cause the northerly connected FTE tube to move downward in the Northern Hemisphere and the southerly connected tube to move duskward in the Southern Hemisphere. In this picture, therefore, an east-west flow reversal would occur across the magnetopause in Fig. 2, the post-noon duskward flow in the magnetosheath becoming a net downward flow on reconnected flux tubes inside the magnetosphere, such as has been found in ISEE 1 and 2 data²⁰. By an unfortunate coincidence,

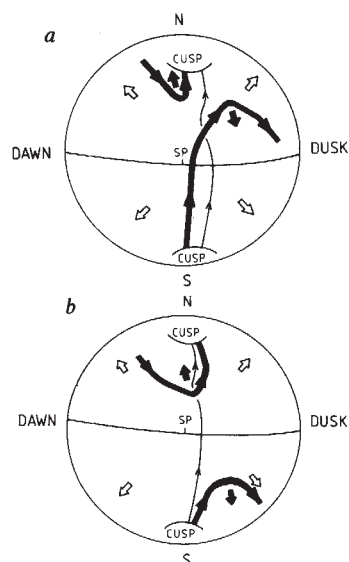


Fig. 3 View of the frontside magnetopause showing reconnection initiated in the northern polar cusp (a) and near the Equator (b) for a southward and duskward magnetosheath field orientation. In both cases, a northerly and a southerly connected flux tube is shown (thick arrowed lines) together with an unreconnected magnetospheric field line underlying the magnetospheric part of one of the flux tubes. The direction of magnetic tension release of the open flux tubes is indicated by the broad solid arrows. Broad open arrows indicate the direction of the external magnetosheath plasma flow. The net motion of the flux tubes is a result of both the ambient magnetosheath plasma velocity and the release of magnetic tension. SP, location of the subsolar point.

however, the flux erosion events in Fig. 2 fall into time gaps between subsequent cycles of HEOS 2 plasma measurements¹, so this point cannot be checked in the data discussed here.

Interpretation of the events in Fig. 2 in terms of northerly connected open flux tubes as shown in Fig. 3b means that the field strength increases inside the FTEs cannot now be explained by extension of the field lines as suggested by Haerendel *et al.*¹. Instead, they may be explained by the field tension effects of the surrounding unreconnected field lines draping over the flux tubes, together with field twisting inside the FTE tubes, as discussed by Paschmann *et al.*⁶. This field twist is implied both by the approximately sinusoidal shape of the B_N signature and the 'anomalous' tangential field deflections sometimes observed inside FTEs^{3,4,21}. 'Anomalous' in this context means that the tangential field inside a FTE does not rotate towards the direction of the field on the opposite side of the magnetopause, but rather rotates away from it. The magnetosheath FTE at 0.26 UT in Fig. 2 is an example showing such behaviour. The tangential field inside this event rotates away from the magnetospheric field orientation. In fact, it has been suggested²¹ that it is the field twist which is primarily responsible for the observed FTE B_N signatures. At present, however, the mechanism responsible for the field twisting inside FTE flux tubes is unknown.

Thus, our preferred picture of the FTEs observed in Fig. 2 is in terms of open flux tubes connected to the Earth's Northern Hemisphere, formed by reconnection southward of the HEOS 2 spacecraft, probably near the Equator. We base this preference first on the assumption that the sense of the bipolar B_N signatures is an indicator of the direction of motion of the FTE flux tubes across the HEOS 2 spacecraft. This assumption is open to question, however, if it is just the field twisting inside the FTE flux tubes that is responsible for the observed B_N signatures. Second, our interpretation also agrees with the results of a recent survey of FTEs, in which combined plasma, magnetic field and energetic particle data from ISEE 1 and 2 were used to determine the hemispheric connection of individual FTE flux tubes¹⁸. Note, however, that without the use of such combined plasma,

magnetic field and energetic particle data, it is impossible to determine conclusively whether the events reported here (Fig. 2) are connected to the Northern or to the Southern Hemisphere, that is, whether the interpretation in Fig. 3a or that in Fig. 3b is the correct one.

At present, statistical studies of FTEs investigating their conditions of occurrence and their properties have only been based on surveys of the ISEE 1 and 2 data^{16,18,22}. The ISEE spacecraft cover the dayside magnetopause in only a limited latitudinal range above and below the magnetic equator. The present results show that it is also possible to identify FTEs in the lower time

resolution HEOS 2 data. As the orbit of the HEOS 2 spacecraft was a highly eccentric polar orbit, with an orbital plane nearly perpendicular to the Earth's equatorial plane (see Fig. 1), this raises the possibility of examining FTEs over different regions of the magnetopause than those covered by ISEE 1 and 2, and of establishing the evolution of FTE signatures as they move along the dayside magnetopause into the tail.

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The martian hemispheric dichotomy may be due to a giant impact

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Mars is divided into two fundamentally different geological provinces of approximately hemispherical extent^{1–3}. The more southerly province is heavily cratered, contains relatively old geological units, and superficially resembles the lunar and mercurian highlands. The northern province is relatively lightly cratered and contains younger geological units, including extensive plains, volcanic edifices, and volcanic calderas. Except for the Tharsis and Elysium regions and other large volcanoes, most of the younger, northern province consists of lowlands, which lie an average of 3 km below the highlands. Lowlands occupy about one-third of Mars. They are separated from the highlands by a distinct scarp or by a sloping transitional zone as much as 700 km wide in which highland materials have been disrupted and partly replaced by lowland deposits (Fig. 1). The transition is characterized by a variety of landforms unknown on other planets. The highlands and lowlands are in isostatic equilibrium across the transitional zone⁴. No generally accepted explanation for the cause of the highland-lowland dichotomy has been proposed although thinning of the lithosphere in the northern hemisphere by internal processes has been suggested⁵. Large impacts, which were a feature of early Solar System history, have also been suggested as an important cause of such early planetary-surface heterogeneities^{3,6,7}. Subcircular lowlands such as Argyre, Chryse, Hellas, and Isidis Planitia and the plains south of Tharsis occupy previously recognized impact basins^{8–10}. We propose here that the largest expanse of lowlands also results from formation of a large impact basin early in Mars' history that has profoundly shaped the planet's surface.

Basins affect planetary surfaces as follows. The impact removes a considerable volume of crustal material and redeposits it on the basin periphery. The loss of mass is partly or fully compensated by isostatic uplift of unexcavated sub-basin

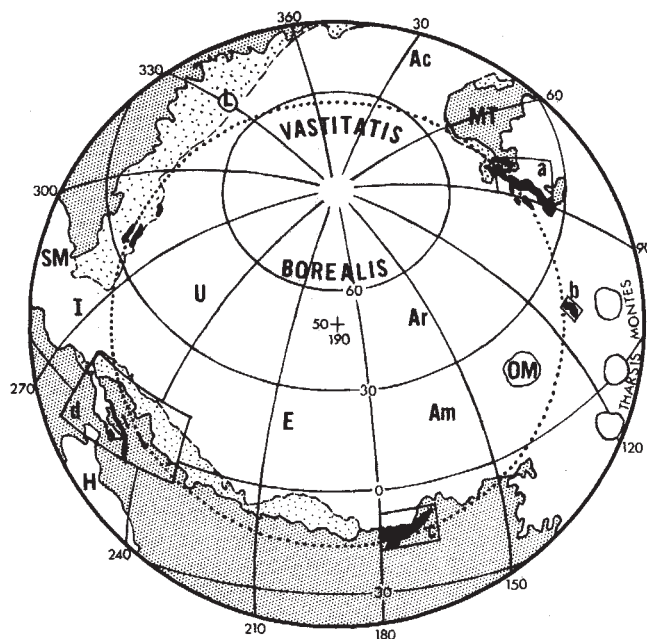


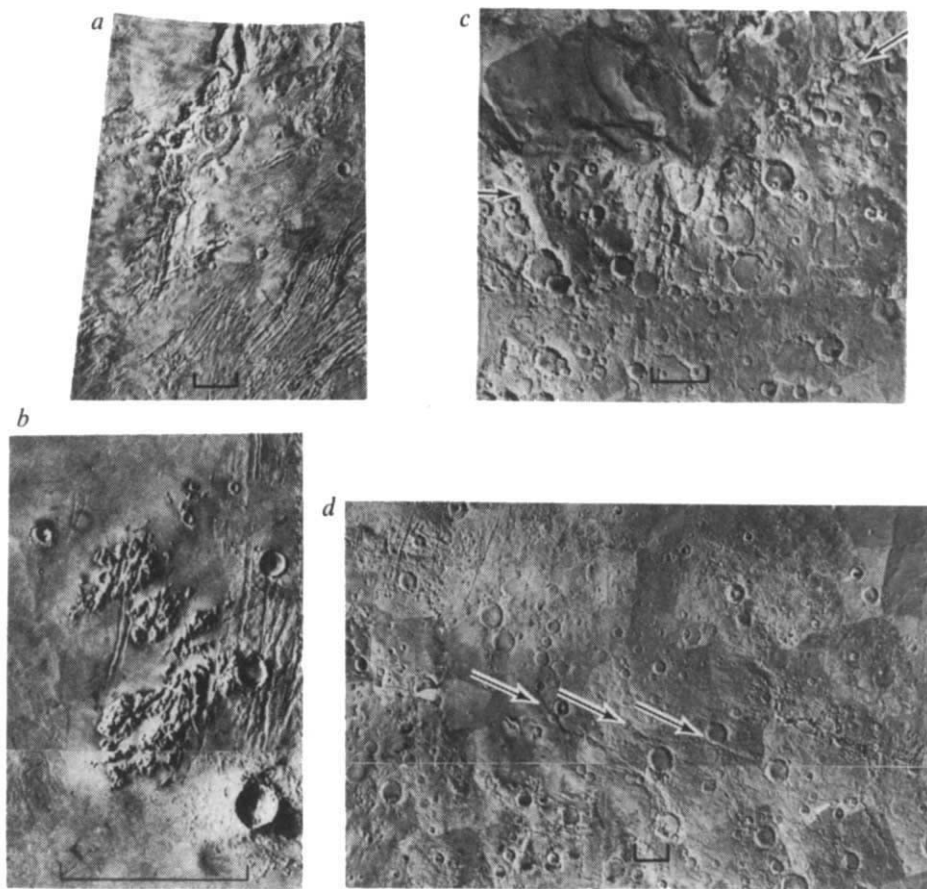
Fig. 1 Polar stereographic projection of part of Mars centred about 50° N, 190° W. Ac, Acidalia; Am, Amazonis; Ar, Arcadia; E, Elysium; H, Hesperia; I, Isidis; SM, Syrtis Major; U, Utopia Planitia; L, Lyot, 215-km ringed basin; MT, Mareotis-Tempe terra; OM, Olympus Mons. Black shading, massifs and narrow plateaus believed part of Borealis basin (including three near E that may be part of inner ring); grey shading, other highland terrain; stippled, with dashed boundary, modified highland terrain in the 'transition zone' (frets, chaos, knobs, channels, and so on); blank, young terrain (including OM and three Tharsis Montes shown separately). Dotted circle indicates inferred rim of Borealis basin.

Fig. 2 (a, b, c, d) outlined.

crustal and mantle material. Because this material is normally denser than the material that was removed, a large depression remains. Terracing or other tectonic subsidence may enlarge the depression. Commonly, the depression is partly filled later in planetary history by lavas rising hydrostatically through the weakened lithosphere from the elevated mantle whenever heat for partial melting is available.

Simultaneously with each basin excavation, two or more mountainous rings are uplifted. The topographic basin rim—

Fig. 2 Parts of Borealis basin rim. *a*, Large, elongate massifs bordering Mareotis–Tempe terra; grabens are Mareotis Fossae; part of mosaic M 2M 39/79 CM centred at 41° N, 83° W. *b*, Highland islands lying in Tharsis bulge between Olympus Mons and Tharsis Montes; mosaic of frames 516A34 and 516A53 centred at 19° N, 112° W. *c*, Arc of massifs (arrows) forming lowland–highland scarp between Elysium Planitia (upper left) and Memnonia region; parts of mosaics M 2M –08/169 CM and M 2M –22/169 CM centred at 12° S, 173° W. *d*, Ridges (arrows) concentric with Borealis basin; massifs and grabens in upper left are parts of Isidis basin; other knobby terrain in upper part of scene is part of highland–lowland transition zone; parts of mosaics M 2M 8/236 CM, M 2M 8/259 CM, M 2M –8/236 CM, and M 2M –8/259 CM centred at 3° N, 246° W. Scale bars, 100 km. Mosaics by US Geological Survey.



which may or may not be the boundary of excavation—encloses most of the basin depression. Additional rings lie inside this rim. The rim and the other rings consist partly of massifs, which on Mars and the Moon characteristically are high mountains having steep slopes, generally irregular outlines, either flat or sharp summits, and usually rough surface structure such as irregular ridges. Narrow plateaus and low sharp scarps also are common around martian basins. Thus, an important test of our hypothesis was to map massifs, narrow plateaus and scarps between latitudes 57° N and 57° S.

Many features thus found that are not parts of a previously known basin lie along a small circle that coincides with the largest contiguous parts of the province boundary (Figs 1, 2). The circle is ~7,700 km (130° of latitude) in diameter (measured along the surface curvature) and is centred at 50° N, 190° W. Westward from 190° W, it follows the hemispheric province boundary between Elysium Planitia and the southern highlands, intersects Isidis Planitia north of Hesperia Planum, follows the province boundary to a point near the small ringed basin Lyot, crosses through Acidalia Planitia, passes through prominent mountains along the northwestern edge of Mareotis–Tempe terra, continues across the Tharsis bulge between Olympus Mons and the three Tharsis Montes, and follows massifs along the province boundary between the Memnonia region and Amazonis and Elysium Planitiae. The area south of the circle contains almost all of the cratered highland province including lowlands concentrated in the previously known basins. Most of the terrain north of the circle is lowland, including Vastitatis Borealis and Amazonis, Arcadia, Elysium, and Utopia Planitiae. We propose that this circular alignment of mountains and scarps defines a 7,700-km basin, which we refer to as the Borealis basin because it encloses Vastitatis Borealis and because of its northern location.

The Borealis basin readily accounts for (1) the largest otherwise unexplained topographic depression on Mars, (2) the corresponding restriction of heavily cratered highlands to outside

the basin rim, that is, either on the basin ejecta or on terrain unaffected by the basin, (3) the existence and concentric orientation of massifs and narrow plateaus not related to other basins, (4) the isolated highland tracts surrounded by lowland plains but lying along the circle (for example, the Mareotis–Tempe terra), (5) the approximate equalization of mass between the lowlands and the highlands, otherwise explicable only by *ad hoc* internal mechanisms, (6) the abrupt scarps between highlands and lowlands, most of which are readily explained as parts of the inward scarp of the basin's rim, (7) the annular transition zone of highland breakup, localized by the topographic rim and inner wall of the basin, and (8) extrusion of many of the lavas in the northern lowlands, because of lithospheric weakening and mantle uplift beneath the basin floor. We believe that a single basin better accounts for the coincidence of all these features than can a number of smaller basins.

Superposition of later smaller basins, however, may explain what departures from circularity do exist. For example, the noncoincidence of the highland breakup zone with the basin trace in Acidalia Planitia may result either from more effective lateral erosion or localization by a smaller basin. The Tharsis province, however, straddles the basin rim and therefore requires an additional cause; localization by another multi-ringed basin has been proposed¹⁰.

A possible objection to the proposed Borealis basin, that such a large impact might destroy the planet, is answered by the fact that the 2,500-km South Pole–Aitken basin, whose existence is well established by enclosure of a lunar–farside depression several kilometres deep by a ring of high massifs, did not destroy the smaller Moon^{11–13}. The radius of South Pole–Aitken (measured along the curvature) is 0.72 times that of the Moon and the radius of the Borealis basin is 1.1 times that of Mars (3,394 km). An even larger lunar basin, the 3,200-km diameter Procellarum or Gargantuan basin, has been hypothesized as the cause of mare concentrations, concentric terra and mare ridges, tectonic structures, geochemical distributions, and many other

features of the nearside¹⁴⁻¹⁶. Basins penetrate less deeply in proportion to their diameters than do small, simple craters¹⁷⁻¹⁹, and therefore inflict less damage than their large diameters might suggest.

The energy required to form a basin as large as Borealis, which may be very roughly calculated from crater-scaling theory, is readily attained by a large impact. The diameter-energy scaling relation is assumed to have the form $D = KE^a h(g)$, where D is the crater diameter, E is the kinetic energy of the impactor, K and a are constants, and $h(g)$ gives the dependence of crater size on surface gravity. For a basin-size impact, 'gravitational strength' will dominate material strength, and we adopt values of $a = 0.29$ and $h(g) = g^{-1/6}$ following Housen *et al.*²⁰ and Gault and Wedekind²¹, respectively. Based on data from cratering experiments and impacts of spacecraft on the Moon, Housen *et al.*²⁰ estimated a value of K of $\sim 7 \times 10^{-2}$ (c.g.s. units). From these values, a kinetic energy of 10^{36} erg is required to excavate a basin 7,700 km in diameter. For an impactor with a density of 3.0 g cm^{-3} and an impact velocity of 24 km s^{-1} (the orbital velocity of Mars), a diameter of $\sim 600 \text{ km}$ is required. Allowing for a velocity as low as 12 km s^{-1} requires a body with a diameter of 950 km. These are reasonable sizes for residual bodies near the orbit of Mars at the end of accretion. For example, Hartmann and Davis²² have calculated the growth rates and final sizes of a family of planetesimals in similar heliocentric orbits. Scaling their results for the Earth linearly to the smaller radius of Mars, the second-largest body near the orbit of Mars following Mars' accretion could plausibly have had a diameter of 1,800 km, the third largest a diameter of 1,100 km, and the tenth-largest a diameter of 380 km. For comparison, the diameters of the three largest asteroids are 1,025, 583 and 555 km (ref. 23). Moreover, the actual values of kinetic energy and impactor size are probably significantly smaller, as the diameter-energy relation was developed for small, simple craters.

The probability that such a large impact occurred on Mars is supported by estimates based on ancient impact rates on the Moon. Basins cover the lunar terra²⁴⁻²⁶. At least 39 basins $\geq 300 \text{ km}$ in diameter were formed before emplacement of the visible mare basalts began about 3,800 Myr ago²⁶. Solidification of the lunar crust roughly 4,200 Myr ago²⁷ sets an approximate maximum age for the oldest observed basins. Therefore, during this 400-Myr interval a population of large projectiles created lunar basins at a minimum rate of 98 per 10^9 yr. Extrapolation of the basins' size-frequency distribution (slope approximately -2 cumulative; based on data from ref. 26) shows that the same population impacting at the same rate would theoretically contain projectiles capable of producing a 7,700-km basin once every 3,500 Myr on the Moon or once every 900 Myr on the 3.8-times larger area of Mars. The probability of such a large basin forming on Mars is even greater if the impact rate was greater before 4,200 Myr ago or if it was greater than the lunar rate²⁸⁻³¹, both of which are likely. Creation of a basin the size of Borealis on Mars is, therefore, consistent with the early impact history of the terrestrial planets.

The existence of Borealis, Procellarum, and other giant planetary basins can be further tested with additional remote observations and, particularly, by data acquired by future spacecraft. A search for massifs, depressions, tectonic patterns, and other features likely to be associated with basins may strengthen or weaken the case for the existence of these hypothesized fundamental elements of planetary structure. Orbital geochemical data may show compositional patterns that result from redistribution of deep-crustal material by major impacts. High-resolution gravity data may reveal patterns such as mascons or concentric positive and negative anomalies that coincide with the proposed basins or, alternatively, that define trends incompatible with the megabasin hypothesis. Probing of deep structure—perhaps by seismometer arrays emplaced by subsurface penetrators—could detect the elevated mantle and thin crust expected beneath large basins.

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NMR of albite-microcline series

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The chemical bonding in silicates has been studied experimentally and theoretically¹. Spinning at 54.72° yields sharp peaks in NMR patterns for ^{29}Si and ^{27}Al (refs 2, 3). Qualitative interpretation of the isotropic chemical shift is useful, but the interrelated effects of framework geometry, cation substitution and hydrogen bonding must be quantified for zeolites. The isotropic chemical shift for ^{29}Si in silica polymorphs is related to the mean secant of adjacent Si-O...Si angles⁴, and the complex NMR patterns of tridymite and uncalcined fluoride silicalite have been interpreted quantitatively (see also refs 5, 6). In some zeolites⁷, the average Si-O-T (tetrahedral) angle is correlated with the isotropic chemical shift for ^{29}Si . The proposed relationships^{8,9} between mean Si-O distance and the isotropic chemical shift for ^{29}Si are poorly obeyed by silica polymorphs⁴, and the cation-oxygen bond strength provides a better correlation than Si-O for a wide range of silicates¹⁰. We show here that the mean secant Si-O-T provides a better guide than Si-O and bond strength for Na,K-feldspars in the series between low albite and low microcline.

To resolve conflicting interpretations of the NMR spectra of alkali feldspars^{2,10}, we traced the three resonances for ^{29}Si across the metastable series between low albite ($\text{NaAlSi}_3\text{O}_8$) and low microcline (KAlSi_3O_8). Albite from Amelia, Virginia, was ion-exchanged to give microcline, and five intermediate compositions were prepared by homogenizing mixtures of albite and microcline at 1,193 K for over 10 days¹¹. The K/(K+Na) ratio, determined by atomic absorption analysis, is probably accurate within 0.02. Unit-cell dimensions are close to those given in ref. 12.

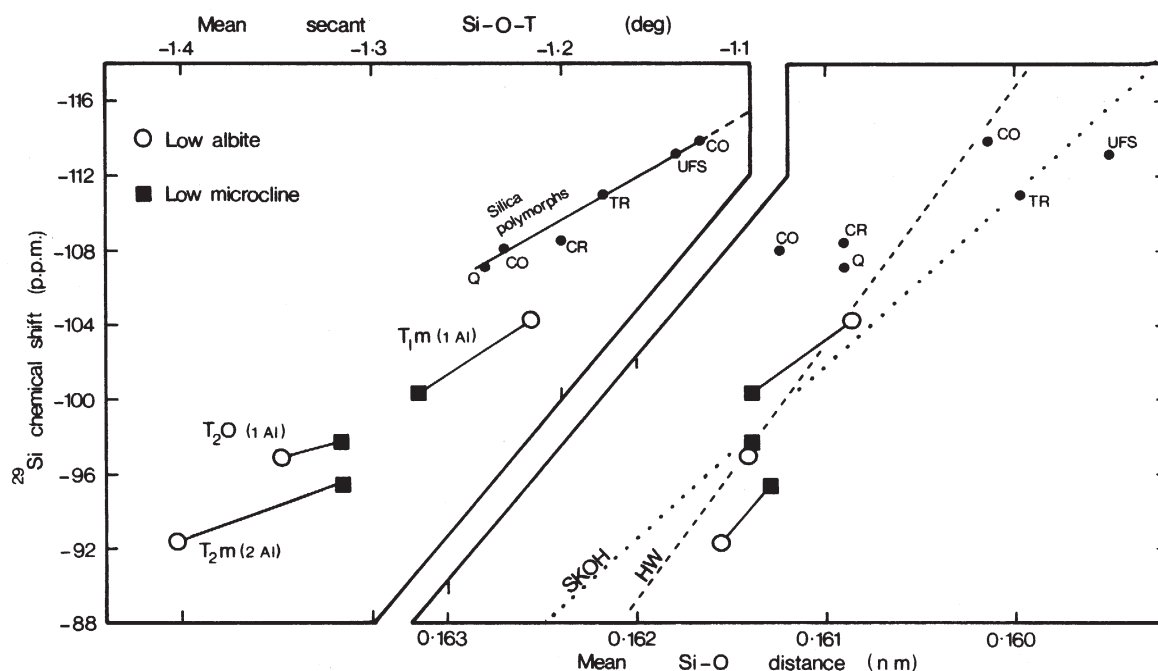


Fig. 1 Relationship between isotropic chemical shift for ^{29}Si and mean secant Si-O-T or mean Si-O distance. The trend for silica polymorphs (upper left) and the labelled dots (upper left and right) are from ref. 4. The dashed and dotted lines (lower right) are from refs 9 and 10 respectively. The data for low albite and low microcline are from Table 2.

The NMR spectra were obtained on a Bruker CXP-200 solid-state and high-resolution NMR spectrometer operating at 4.7 T. A standard ^{13}C cross-polarization magic-angle spinning accessory probe was tuned to 39.7 MHz for ^{29}Si -NMR and 52.13 MHz for ^{27}Al -NMR. The ^{29}Si -NMR chemical shifts are referred to external TMS (tetramethylsilane) and the ^{27}Al -NMR chemical shifts to external $\text{Al}(\text{H}_2\text{O})_3^{3+}$ in saturated $\text{Al}(\text{NO}_3)_3$ aqueous solution. Changes of only a few hertz back and forth were recalibrated daily. The Andrew-Beam single-bearing rotors are made of Delrin and were spun with dry nitrogen. The sample spinning rate of 3,500 Hz for ^{29}Si -NMR (88 p.p.m. for ^{29}Si at 39.7 MHz) obviated spinning sidebands. For ^{27}Al -NMR the sample spinning rate was increased to ~ 4 kHz to move the spinning sidebands away from regions of interest. A typical ^{29}Si -NMR spectrum was obtained from Bloch decay experiments of 5,000–8,000 co-added free induction decays produced by 2- μs pulses followed by a 10.2-s relaxation delay; 20.4 s caused no change. For ^{27}Al -NMR, typically 500 Bloch free induction decays were accumulated using 2- μs pulses and a 1-s delay between successive pulses. The spectra generally show spinning sidebands and lineshapes suggestive of incomplete removal of second-order quadrupole coupling.

Peak positions (Table 1) of ^{29}Si for the end members are consistent within experimental error with those reported elsewhere^{2,9,10,13,14}. The continuity of each peak across the series demonstrates an incorrect labelling for at least one peak in ref. 10, and resolves the uncertainty in ref. 2. The splitting of the peaks decreases nonlinearly from albite to microcline, as do the cell dimensions^{12,15}.

The three peaks were assigned unambiguously to the three structural positions T_{1m} , T_{2O} and T_{2m} for Si in the feldspar framework by use of mean secant Si-O-T (Table 2, Fig. 1). Accurate neutron diffraction data for Amelia albite¹⁶ are consistent with various X-ray data of lower accuracy. Early X-ray diffraction data for Pellotsalo microcline¹⁷, used in ref. 10, are less precise than those for a series of partly disordered K-feldspars from the Adamello Massif¹⁸, and the angles and distances for microcline in Table 2 were obtained by 12% extrapolation towards maximum low microcline past the data for specimen CAIE. Because of the positive trend for the silica polymorphs⁴, the six possible assignments between three framework sites and three resonances were examined for close-

ness to an overall positive trend for the alkali feldspars. Only one choice is possible for low albite, whereas there are two choices for low microcline because two sites have the same value for mean secant Si-O-T; one of these choices matches the choice for albite. The chosen assignment has the smallest value of the chemical shift for the T_{2m} site linked via oxygens to 2 Al. Lippmaa *et al.*² also assigned the most paramagnetic resonance to Q4 (2 Al) tetrahedron but labelled it incorrectly as T_{2O} .

The scatter of data is greater for a plot of isotropic chemical shift of ^{29}Si versus mean Si-O distance (Fig. 1, right), but the general positive trend indicates the same assignment for the feldspar resonances as in Fig. 1 (left). There are deviations up to 4 p.p.m. from the trends proposed by Higgins and Woessner⁹ and by Smith *et al.*¹⁰.

Although there is a general positive trend in Fig. 2 between the isotropic chemical shift for ^{29}Si and the sum of cation bond strengths¹⁹ for the four oxygen atoms of a tetrahedron, the negative relation for T_{1m} is unhelpful in making an assignment. Without knowledge of the continuity of the resonance across the microcline-albite series, mislabelling could occur (as in ref. 10). Actually, the calculation of cation bond strengths is uncertain for feldspars because of uncertainty in the coordination of the non-framework cation to the framework oxygens¹⁵. The bond strengths at the oxygen atoms (Table 1) were calculated on the

Table 1 Isotropic chemical shifts for ^{29}Si and ^{27}Al

Sample	K/(K + Na)	^{29}Si			^{27}Al
Amelia albite	0.0	-92.3,	-96.9,	-104.3	+40.1†
8,205	0.17	-93.0,	-97.1,	-104.0	+44.1
8,207	0.33	-93.5,	-97.1,	-102.8	+46.9
8,047	0.49	-94.2,	-97.0,	-101.3	+48.0
8,204	0.65	-94.6,	-97.0,	-100.8	+50.1
8,206	0.83	-95.0,	-97.3,	-100.7	+47.3
		-94.9*,	-97.6*,	-100.6*	
71,104	1.00	-95.6,	-97.6,	-100.6	(+47.5†)
		-95.4*,	-97.8*,	-100.4*	

* From curve resolution; all others from top of peak.

† Peak maximum of incompletely narrowed quadrupole pattern. The peak maxima of intermediate compositions correspond more closely to the isotropic chemical shift. The peak position for 1.00 K/(K + Na) was obtained for a microcline from Asbestos, Quebec.

Table 2 ^{29}Si chemical shifts and atomic parameters

Specimen	Chemical shift (p.p.m.)	Assignment	Mean Si-O (nm)	Si-O-T angles (deg)	Mean secant Si-O-T	Bond strengths of oxygen atoms
Low albite	-92.3 (-92.5)*	T ₂ m	0.16156	130.1, 161.2, 129.9, 133.9	-1.403	2.126, 2.085, 1.949, 2.013 (8.173)
	-96.9 (-96.7)*	T ₂ O	0.16141	130.1, 139.7, 135.8, 151.8	-1.348	2.126, 2.043, 2.032†, 2.101 (8.302)
	-104.3 (-104.2)*	T ₁ m	0.16087	141.5, 161.2, 135.8, 151.8	-1.216	2.103, 2.085, 2.032†, 2.101 (8.321)
Microcline	-95.4	T ₂ m	0.1613	138.0, 155.6, 130.8, 140.6	-1.317	2.116‡, 2.130, 1.996, 1.987 (8.228)
	-97.8	T ₂ O	0.1614	138.0, 150.9, 131.3, 142.4	-1.317	2.116‡, 2.009, 2.046, 2.107 (8.278)
	-100.4	T ₁ m	0.1614	144.4, 155.6, 131.3, 142.4	-1.276	2.117, 2.130, 2.046, 2.107 (8.400)

* Values in parentheses are results from ref. 2.

† Excludes Na atom at 0.3266 with bond strength of 0.033.

‡ Excludes K atom at 0.3429 with bond strength of 0.048.

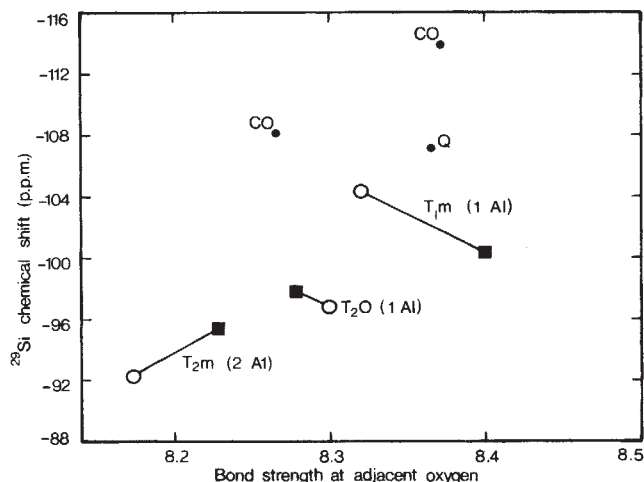


Fig. 2 Relationship between isotropic chemical shift for ^{29}Si and the combined bond strength for the four adjacent oxygen atoms (data from Table 2).

assumption that Na is bonded to seven oxygens at 0.237–0.330 nm in low albite¹⁶, and that K is bonded to nine oxygen atoms at 0.273–0.335 nm in microcline^{17,18}. Addition or subtraction of an oxygen will change the bond strength by at least 0.03–0.05, but there is no obvious way to reverse the negative relationship for T₁m.

The single resonance for ^{27}Al is assigned to the T₁O tetrahedral site. The peak shift of about 7 p.p.m. as K replaces Na in low albite shows a positive trend with the mean secant Al–O–Si angle (low albite¹⁷, -1.398; microcline, extrapolation from ref. 18, -1.300). Hence, it appears that the mean secant Al–O–T angle may be worth exploring as a possible predictor for the isotropic chemical shift of ^{27}Al .

We conclude that the mean secant Si–O–T is a better predictor for the isotropic chemical shift of ^{29}Si in anhydrous frameworks than either mean Si–O or the combined bond strengths on the adjacent oxygen atoms. Nevertheless, assignment of resonances to framework nodes must be made cautiously even from mean secant Si–O–T, and comparison with ion-exchanged species may be desirable. Further work on Rb-exchanged feldspars and on zeolites with accurately known hydrogen positions is in progress.

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Flexure of the continental lithosphere beneath Apennine and Carpathian foredeep basins

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The formation and development of foredeep basins adjacent to marginal fold and thrust belts are thought to be the result of flexural loading of the foreland lithosphere by migrating thrust sheets¹, and this concept has formed the basis for many recent studies of the deformation of the continental lithosphere and consequent evolution of foreland basins^{2–7}. The preservation of foredeep basins, after the associated mountain belt has been greatly reduced by erosion, suggests that an additional load, independent of the topography, must act on the lithosphere of the subducted slab⁶. To determine whether such an additional load might be present in some very young orogenic belts, we constructed transects through the Apennine and outer (flysch) Carpathian thrust belts of the Mediterranean region (Fig. 1). Using a simple elastic model, which assumes a semi-infinite (broken) elastic plate of constant flexural rigidity overlying a weak fluid, we show that the topographic loads present along these transects are insufficient to cause the observed plate deflection. This suggests that there is an additional load (or downward force) acting on the plate to produce the observed plate deflection beneath the foredeep troughs. We suggest that this extra load resulted from either the negative buoyancy contrast between the subducted slab and the surrounding asthenosphere, or modification of the crustal structure within the overriding plate due to the initiation of backarc rifting in the hinterland.

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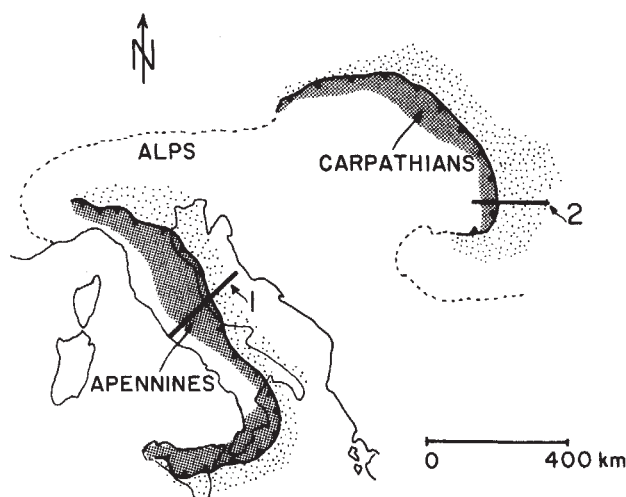


Fig. 1 Location of the transects across the Apennine and Carpathian thrust belts (dark lines). The Apennine and outer Carpathian thrust belts are indicated by shading and the approximate position of the thrust fronts are indicated by dark barbed lines. The light continuous line represents the present coast line, and the light dashed line indicates the most external, pre-Miocene thrust faults in the Alps, South Carpathians and Balkan mountains. Stippling represents approximate extent of foredeep deposits for the Apennines and Carpathians.

Both the Apennine and Carpathian belts consist of allochthonous thrust and fold complexes transported over a relatively stable, continental foreland with a total of several hundred kilometres of late Cenozoic shortening⁸⁻¹⁰. External to (east of) the Apennine thrust belt, an asymmetric foredeep trough contains locally more than 8 km of Pliocene and Quaternary sedimentary rocks (CNR Italian Geodynamics Project, map of Pliocene isobaths, in preparation). External to (north and east of) the Carpathian belt, a foredeep trough contains up to 9 km of Upper Miocene, Pliocene and Quaternary sedimentary rocks¹¹⁻¹³. In both cases, the modern foredeep trough developed contemporaneously with the last stages of thrusting. Moreover, both thrust belts appear to have developed synthetically with respect to subduction, as indicated by intermediate and deep earthquakes and arc-related volcanic rocks¹⁴⁻¹⁸.

The deflection of the forelands during nappe emplacement can be determined from the present depth to the base of Pliocene (Apennines) and Miocene (Carpathians) sedimentary rocks within the foredeep troughs, because before these times shallow or non-marine conditions prevailed in the areas occupied by the modern foredeep troughs (and probably for 50–100 km further west^{8,15,19,20}). In the Apennine foredeep this interpretation is complicated by the rapid changes in sea level during the Messinian.

To analyse the deflection of the foreland lithosphere, the foreland was modelled as a broken elastic slab with a stress-free end and uniform flexural rigidity, overlying a fluid asthenosphere ($\rho_m = 3.3 \text{ g cm}^{-3}$). The slab end probably represents a zone beyond which the subducted lithosphere is so weak that the transmission of bending stresses through the slab is greatly reduced, either because of heating or fracturing of the slab during subduction. Because both the Apennine and Carpathian forelands appear to be near thermal equilibrium and mostly unaffected by recent tectonic events, and because their geometry does not vary significantly along-strike near the transects used (Fig. 1), we have assumed two-dimensionality throughout this study.

In our first analysis of the transects shown in Figs 2 and 3, we assumed that the observed topography represents the only load acting on the lower plate lithosphere. The load due to that part of the thrust complex and foredeep sediments that are below sea level was considered to be a part of the buoyancy

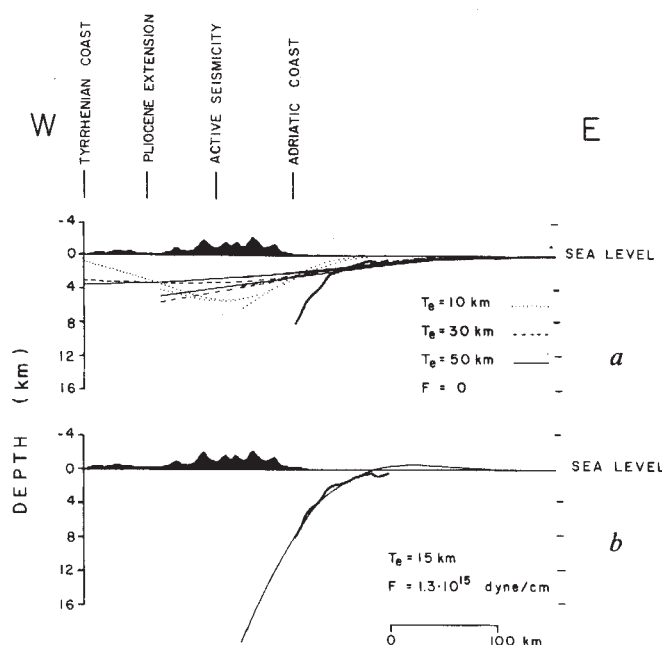


Fig. 2 Transect across the Apennine thrust belt and Adriatic foreland showing topography (in black) and observed thickness of Pliocene to Recent sedimentary rocks within the foredeep trough (heavy line). The depth to base Pliocene was taken from the CNR Italian Geodynamics Project map of depth to base Pliocene (in preparation). These depth determinations are based on drill hole and seismic lines of AGIP. The thrust front on this transect is located just east of the Adriatic coast. *a*, Set of nine curves showing the calculated deflection of the lower (Adriatic) plate for effective elastic plate thicknesses (T_e) of 10, 30 and 50 km and three different choices for the lateral position of the slab end. In each case the load was assumed to be equal to the observed topographic load plus the weight of the infilling rocks above the calculated surface of the lower plate and below sea level. *b*, Calculated deflection of the lower plate assuming an elastic plate thickness of 15 km and an additional vertical line force, F (equivalent to a point force in two-dimensional analysis) of $1.3 \times 10^{15} \text{ dyn cm}^{-1}$ downwards. (See Fig. 1 for location [transect 1].)

term in the differential equation governing elastic behaviour, and for simplicity the density of the nappes and foredeep sediments were considered equal ($\rho_s = 2.7 \text{ g cm}^{-3}$). Although unrealistically high for the over-pressured foredeep sediments, this density gives a maximum estimate of the load and hence of the subsequent deflection. Thus we need only specify the observed topography present along each transect, and compare the resulting plate deflection with that observed.

The equation used to describe the behaviour of the elastic slab is:

$$D \frac{d^4 w(x)}{dx^4} + (\rho_m - \rho_s) g w(x) = \rho_s g h(x)$$

where D is the flexural rigidity of the slab, x is horizontal distance along the slab, $w(x)$ is the plate deflection at x , $h(x)$ is the topographic elevation at x , ρ_m is the mantle density and ρ_s is the sediment density and the density of the mountains. The boundary conditions at the stress-free slab end are:

$$\frac{d^2 w(x)}{dx^2} = \frac{d^3 w(x)}{dx^3} = 0 \text{ at } x = 0$$

and at the other end: $w(x) \rightarrow 0$ as $x \rightarrow \infty$.

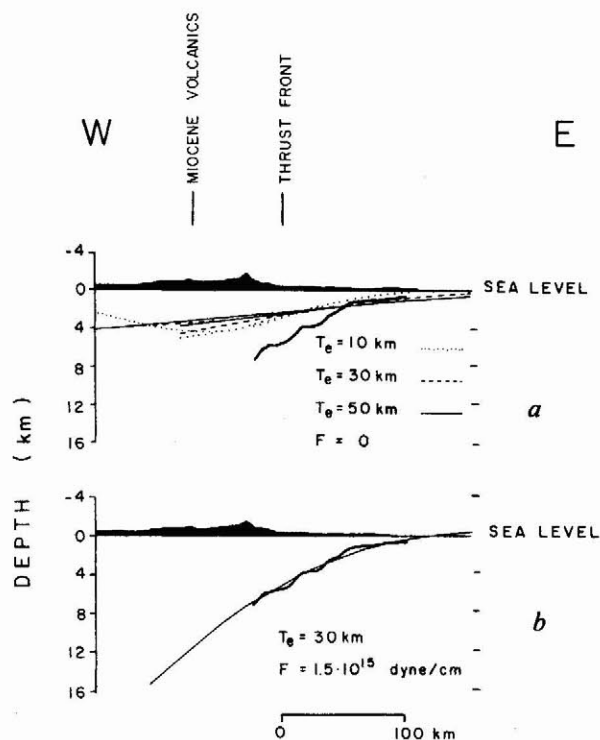


Fig. 3 Transect across the Carpathian thrust belt and European foreland showing topography (in black) and observed thickness of Miocene to Recent sedimentary rocks within the foredeep trough (dark line). Depth to base Miocene taken from ref. 11. *a*, Set of six curves showing the calculated deflection of the lower (European) plate for elastic plate thickness of 10, 30 and 50 km, and two different choices for the lateral position of the slab end. As before, the load was assumed to be equal to the topographic load plus the weight of the infilling rocks above the calculated surface of the lower plate and below sea level. *b*, Calculated deflection of the lower plate assuming an elastic plate thickness of 30 km and an additional vertical line force (equivalent to a point load in two-dimensional analysis) of 1.5×10^{15} dyn cm⁻¹ downwards.

Figures 2a and 3a summarize the calculated deflection of the foreland basement caused by the observed topographic loads for effective plate thicknesses of 10 km, 30 km and 50 km (or equivalently, flexural rigidities of $D = 6 \times 10^{28}$, 2×10^{30} and 9×10^{30} dyn cm), and several different positions of the slab end. It can be seen that the maximum predicted depths are only about one-half to one-third that of the observed Apennine and Carpathian foredeeps, regardless of the flexural rigidities assumed. Neither increasing nor decreasing the assumed rigidities improves the fit between observation and prediction; increased rigidities tend to decrease plate curvature, while decreased rigidities tend to increase plate curvature but also localize the deflection almost directly beneath the topographically high part of the belt.

We conclude, therefore, that the topographic load represented in the transects across the Apennines and Carpathians (Figs 2a and 3a) is insufficient, by about a factor of two, to cause the observed deflection of the continental basement beneath the foredeeps. This result demands the existence of an additional subsurface load (or force) which acts on the subducted plate and isostatically maintains the deflection of the observed foreland basin. Such a load might be due to the negative buoyancy of the subducted slab relative to the surrounding asthenosphere, or to back-arc rifting of the overriding plate that replaces light crustal material with denser mantle material. Regional isostatic adjustment of the overriding plate, as a result of this crustal

thinning, also flexurally deforms the subducted slab. Regardless of the exact geological significance of the subsurface load, its magnitude can be estimated by applying an additional vertical force at the free slab end such that the calculated deflection approaches that of the observed foredeep.

Figures 2b and 3b show the calculated deflection of the Apennine and Carpathian forelands that best fit the observed deflections, assuming that in addition to the topographic load, a vertical force is applied at the slab end. In general, the slope and curvature of the calculated deflection are very sensitive to plate rigidity, the position of the free end, and the magnitude of the applied force. The best fitting solutions in Figs 2b and 3b (chosen by eye) correspond to an effective plate thickness of 15 km and a subsurface (vertical) force of 1.3×10^{15} dyn cm⁻¹ for the Apennine transect, and of 30 km and 1.5×10^{15} dyn cm⁻¹ for the Carpathian transect. Slope, curvature and total displacement are all in good agreement with observation, but it is important to note that the rigidity estimates determined here only represent lower bounds. The large degree of curvature observed in the foreland plate may, in part, be related to plastic yielding and so assuming a constant rigidity plate is only a first-order approximation.

The applied vertical force (in addition to the topographic load) is roughly equal to the topographic load present along the transects. If associated with a density difference between subducted lithosphere and asthenosphere of $+0.1$ g cm⁻³, it is equivalent to the negative buoyancy force generated by a slab with cross-sectional area of $\sim 1,300$ – $1,500$ km² in a vertical plane perpendicular to the trench axis. It is also in reasonable agreement with the vertical trench force ($\sim 5 \times 10^{15}$ dyn cm⁻¹) obtained by Davies²¹.

This study does not imply that the topographic load of all thrust belts is insufficient to produce their respective foredeep troughs. Indeed, results from the Himalayan mountain/Ganges Basin^{6,7} and preliminary results from the Andes (H. Lyon-Caen, in preparation), indicate that the topographic loads present in these mountain belts are sufficiently large to account for the deflection of the plate beneath the foredeep trough and along the trench axis. However, it is clear that in some thrust belts (such as the Appalachian and Molasse basins) the topography is inconsistent with the depth of the foreland basin.

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Melange production and the importance of shale diapirism in accretionary terrains

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Although melanges are widely believed to form in subduction zones, there is little agreement as to the mechanism by which the characteristic chaotic aspect of a melange develops^{1,2}. Debate centres on the relative effects of gravity sliding down the inner trench slope and shearing along thrust faults during offscraping or underplating^{2,3}. The results of deep sea drilling and mapping of modern accretionary terrains⁴ combined with geophysical investigation of outer arc regions favour the thrust or fold-packet model^{1,4-8} of accretion, although some melange deposits exposed on land appear to fit an olistostrome model better^{1,9}. Shale diapirs and mud volcanoes are abundant in many modern accretionary terrains¹⁰⁻¹², and probably form as a result of rapid overloading caused by tectonic thickening^{4,13}. An investigation of extensive melange zones in northern Irian Jaya has now shown that they are the product of shale diapirism, which suggests that shale diapirism is an important process in the tectonic mixing of accretionary terrains.

Recent mapping in northern Irian Jaya by the Indonesia-Australia Irian Jaya Geological Mapping (IJGM) Project led to the discovery and investigation of a discontinuous belt of melange extending from Sarera Bay to the Papua New Guinea border¹⁴ (Fig. 1). Northern Irian Jaya consists entirely of thick Neogene to Pleistocene turbidites which were laid down on a basement of ultramafic rocks and Palaeogene island arc and ocean-floor volcanic rocks (Auwewa Volcanics) in a rapidly subsiding trough, formed adjacent to the northern margin of Australian continental crust. Downbuckling began in the middle Miocene (when the Makats Formation was deposited) and appears to have reached a maximum between the latest Miocene and the mid-Pleistocene, during which time over 7,000 m of turbidites (Mamberamo Formation) were deposited. In the mid-

Pleistocene the sedimentary rocks and the underlying basement were deformed and uplifted before thin paralic sediments (Kukunduri Formation) were laid down over parts of the region. Deformation has continued since the mid-Pleistocene and is concentrated along a zone of disruption 400 km long and nearly 100 km wide north of the northern margin of Australian continental crust (Fig. 1). The zone is characterized by complex faults and folds; shale diapirs constitute over a third of the zone¹⁴.

The diapirs have a characteristic topographical expression of hilly terrain with low relief scarred by landslips. The stream pattern is very irregular and in places the landscape is littered with blocks of resistant limestone and basic volcanic rocks. Mud volcanoes, mud flows and mud seeps cap the diapirs in places and form extensive fields¹⁴ (up to 50 km long and 25 km wide) or are aligned along structural trends¹⁵. Individual mud volcanoes range from 3 m to 2.5 km in diameter and reach a maximum height of about 110 m. The larger volcanoes are clearly visible on aerial photographs and by satellite imagery. The presence of active mud volcanoes, the ubiquitous landslips, and upwarping of Recent river gravels by the diapirs¹⁴ show that the diapirism is currently active throughout the region.

The diapirs consist of pervasively sheared mudstone (scaly clay) containing chaotically distributed angular to rounded clasts ranging in diameter from a few millimetres to 10 m or more (Fig. 2). The clasts consist of mudstone, siltstone and shale (native blocks), and sandstone, conglomerate, limestone, volcanic rocks and serpentinite (exotic blocks). The diapiric material is therefore melange^{2,16}, which compares favourably in texture and fabric with tectonic melange^{2,3,17} (Fig. 2).

The source rock for the diapirs appears to be the oldest sedimentary rocks of the region, the middle Miocene Makats Formation, as the diapiric mudstone matrix contains abundant middle Miocene foraminifera whereas the Mamberamo Formation is latest Miocene to mid-Pleistocene in age. The presence of exotic blocks of limestone (Eocene (?) to early Miocene), volcanic rocks and serpentinite from deeper stratigraphical levels attests to structural inversion (over-folding or thrusting) before diapir initiation. The observation of shale diapirs cutting overturned fold limbs indicates that structural overturning of the surrounding coherent sedimentary sequence on inclined to recumbent folds, with associated cleavage formation¹⁴, also occurred before diapir intrusion.

The Makats and Mamberamo Formations were deposited as submarine fan turbidites in a trough developed adjacent to the northern edge of the Australian continent. The trough formed by downwarping of oceanic crust as a result of compression

Fig. 1 Distribution of diapiric melange zones and active mud volcano fields, northern Irian Jaya. Most of the diapirs are currently active, but others are probably extinct.

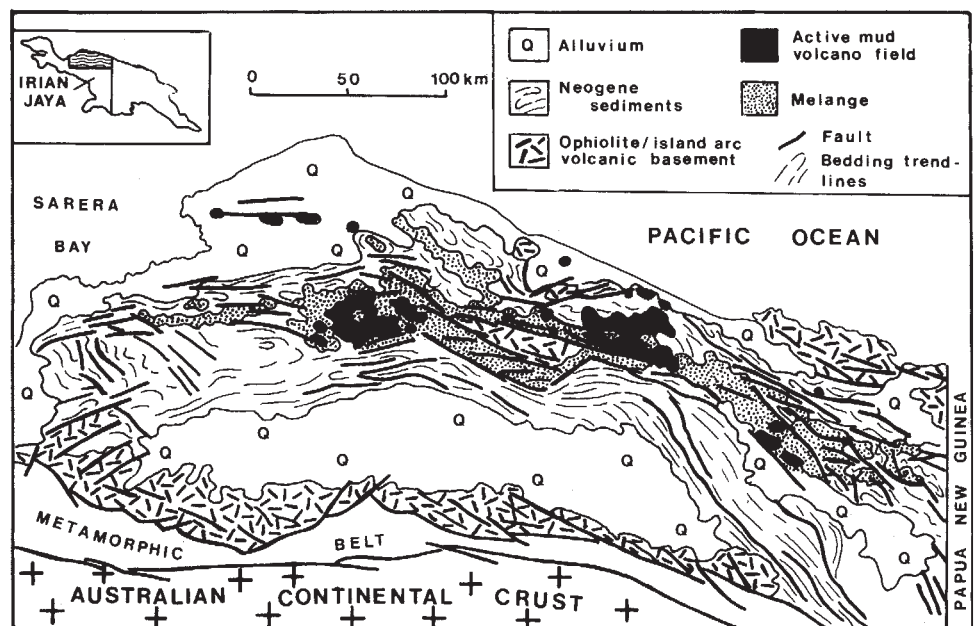




Fig. 2 Native blocks of siltstone dispersed in a scaly clay matrix of an active diapir. Note the polished surfaces of the large clast, the preferred orientation and sheared clasts, typical melange structures.

between the Australian and Pacific plates, but there is little evidence that underthrusting was occurring at this time. In any event the trough sedimentary fill was derived from the uplifted Australian continent to the south. The sedimentary sequence is therefore considered to be *in situ* and not accumulated by offscraping or underplating from an oceanic plate. The marked similarity between the northern Irian Jaya diapiric terrain and accretionary terrains is probably the result of subsequent deformation associated with plate convergence.

Because of the worldwide occurrence of mud volcanoes and shale diapirs in many modern accretionary terrains^{10-12,18}, and the remarkable similarity between rock structure and fabric of the northern Irian Jaya diapirs and ancient accretionary terrain melange, we suggest that shale diapirism is an important, and until now overlooked, process in the development of the internal structure of accretionary terrains. The conditions in accretionary terrains, where thickened and overpressured, water-saturated sediment is disrupted during underplating or offscraping, are ideal for the initiation of diapirism (for example, South Barbados Ridge Complex^{11,19}). We therefore think it likely that the chaotic aspect of melange in accretionary terrains has resulted not only from shearing during underplating and offscraping but also from mixing and shearing by diapirism.

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First estimate of annual mercury flux at the Kilauea main vent

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Mercury is the principal metal emitted in vapour form by volcanoes and other natural geothermal sources. Direct evidence for the release of mercury at volcanic, fumarolic and hydrothermal sites has been obtained in Hawaii, Iceland, Antarctica and the Pacific north-west¹⁻⁶, and the relationship of mercury to volcanic activity elsewhere has been inferred from the analysis of indicator plants^{7,8}. The relation of volcanogenic mercury to total atmospheric content has been a matter of some uncertainty. From 1971 to 1980, mercury and sulphur dioxide analyses were carried out on air samples collected immediately downwind of Halemaumau, the Kilauea main vent, in Hawaii. From these measurements, a Hg/SO₂ ratio of 0.51×10^{-3} was derived. As reported here, applying this ratio to the recently determined sulphur dioxide mass output of Halemaumau, yields a calculated mercury flux of 2.6×10^8 g annually. This value is consistent with recent evidence⁶ suggesting that volcanogenic mercury contributes significantly to the atmospheric total.

In 1977, the US National Academy of Science's Environmental Studies Board ranked its mercury flux estimate of 2×10^7 g yr⁻¹ as "insignificant"⁹. Even Anderson's higher value, $\sim 10^8$ g yr⁻¹ (ref. 10), is small (0.5%) compared with estimates of $\sim 2 \times 10^{10}$ g yr⁻¹ for planetary sources of atmospheric mercury which have been ascribed principally to continental degassing and secondarily to anthropogenic and other sources^{11,12}.

Recently, Varekamp and Buseck proposed a value for volcanogenic Hg output of 10^9 to 7×10^9 g yr⁻¹ (ref. 6), an estimate based on aerial measurements during September 1980 in the Mt St Helens plume. Their data, which yielded a Hg/SO₂ ratio of 10^{-3} , were then applied to the SO₂ flux data of Stoiber and Jepson¹³. In their communication, however, Varekamp and Buseck noted that results from other volcanoes, Kilauea included, might require that their ratio of 10^{-3} be reduced to 10^{-5} , or even 10^{-6} .

Our own measurements in the air corridor up to 2,500 m between the islands of Hawaii and Oahu, indicated that at least 10^6 g of mercury were present in the air ($\sim 10^{12}$ m³) sampled during each of seven 2-h flights¹⁴, again suggesting that previous values of Hg flux, have been far too low. We now present the results of measurements at Kilauea which suggest that a Hg/SO₂ ratio differing only slightly from the Varekamp-Buseck value of 10^{-3} , may be applied to Kilauea also.

Mercury and sulphur dioxide sampling has been carried out in our laboratory since 1971; standard collecting and analytical procedures have been used¹⁴⁻¹⁷. Portable samplers and impinger traps were located side-by-side and operated at field sites within the same 2-4-h time intervals. Sample flow rates were 1-2 l min⁻¹. Stations were always located downwind of the Kilauea

Table 1 A chronological record of downwind Hg and SO₂ emissions at Kilauea main vent, Halemaumau caldera

Sample period	No. of stations	Total no. of analyses	Sample composition ($\mu\text{g m}^{-3}$)		Hg/SO ₂ $\times 10^3$
			Hg	SO ₂	
August 1971	4	10	36 $\pm$ 11	24,180 $\pm$ 2,340	1.40
January 1972	3	8	7.0 $\pm$ 2.3	9,620 $\pm$ 1,560	0.70
August 1975	3	6	1.0 $\pm$ 0.2	5,980 $\pm$ 1,820	0.17
June 1976	4	10	6.4 $\pm$ 2.1	18,980 $\pm$ 4,420	0.34
July 1977	4	9	4.1 $\pm$ 0.8	10,400 $\pm$ 2,080	0.39
August 1977	3	8	0.2	700 $\pm$ 182	0.29
October 1977	4	10	50 $\pm$ 8	52,000 $\pm$ 5,980	0.96
August 1978	4	10	2.8 $\pm$ 1.1	13,260 $\pm$ 2,080	0.21
August 1979	2	5	6.0 $\pm$ 0.8	13,780 $\pm$ 3,900	0.44
February 1980	4	10	2.6 $\pm$ 2.0	14,560 $\pm$ 3,640	0.18
Total:	35	86	11.6 $\pm$ 2.8	16,346 $\pm$ 2,500	0.51

Sample period; downwind stations and total number of replicates per data set are given. Mean values $\pm$ s.d. are presented.

caldera's main vent at Halemaumau (see Fig. 1; ref. 18) usually at, or within, 30 min of the nearest edge. For any one sampling period, the specific location was, of course, determined by the immediate wind direction observed. Samples taken during heavy rainfall have been omitted, but some measurements include periods of elevated activity (October 1977). Results are tabulated for 10 separate periods identified by month and year; two to four sample stations were occupied on each date. For each of these sample stations two to three replicate analyses were made. In all, 86 analyses were carried out each for mercury and sulphur dioxide at 35 sample stations.

A decade of observations shows that, as was found at Mt St Helens over a far shorter period, both mercury and sulphur dioxide emissions vary with time (Table 1), the amplitude of variation being greater for mercury, that is 250-fold compared with 70-fold for sulphur dioxide.

Following Varekamp and Buseck, we have calculated the ratio Hg/SO₂ for each sample period. The mean ratio is $0.51 \pm 0.31 \times 10^{-3}$ with a range of $0.21\text{--}1.40 \times 10^{-3}$. This compares well with Mt St Helens ratios of $0.5\text{--}1.6 \times 10^{-3}$, averaging 1.4×10^{-3} . Thus, there is substantial agreement to one order of magnitude in Hg/SO₂ ratios for the two volcanoes.

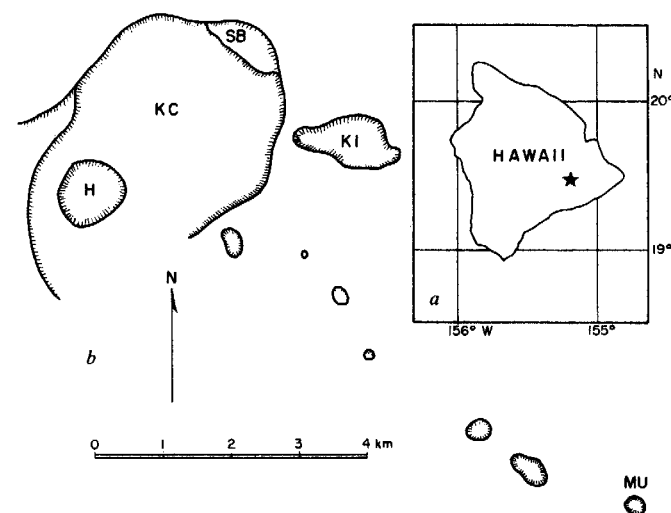


Fig. 1 Kilauea volcano, Hawaii. The geographical locations of the island (a) and of the Kilauea summit (star) are shown. b, Detail of the summit caldera (KC), together with associated geological features, including Kilauea Iki (KI), the Sulfur Bank fumaroles (SB) and Mauna Ula crater (MU). The site of our measurements was the periphery of Halemaumau (H) firepit, the main vent of Kilauea. When, as is usual, the trade winds blow from the north-east, 'downwind' (see text) refers to the south-west arc of Halemaumau. Otherwise, downwind refers to any sites along the circumference opposite the wind direction.

Between June 1979 and June 1982, 103 correlation spectrometry (Cospec) measurements at the Halemaumau vent yielded a mean SO₂ output of $1.50 \pm 0.6 \times 10^8$ g per day (P. Greenland and R. Decker, personal communication). Data taken during phases of active eruption were excluded. Using our mean ratio and this approximate value for sulphur dioxide flux at Halemaumau yields a daily Hg output of $\sim 7.7 \times 10^5$ g, or 2.6×10^8 g annually. Thus, we find that the Kilauea main vent alone releases mercury at a rate consistent with Varekamp and Buseck's higher value of 10^6 g per day or 3.7×10^8 g yr⁻¹. Furthermore, Stoiber *et al.*¹⁹ have reported a yield from six Central American volcanoes of 3.5×10^8 g yr⁻¹. Thus, only eight of the many currently active terrestrial volcanoes²⁰ release Hg at a rate of $\sim 10^9$ g yr⁻¹, which represents 5% of the total flux.

We conclude that the contribution of volcanoes and associated geothermal sources to the atmospheric mercury burden has been underestimated and requires further evaluation. Even relatively sizeable errors in these determinations would not alter that conclusion.

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Photoelectrochemical pumping of enzymatic CO₂ reduction

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The need for renewable resources of carbon-based fuels and chemicals has focused on methods by which carbon dioxide could be utilized as the initial substrate. However, most of these methods have tended to be inefficient, both in terms of energy losses involved during CO₂ fixation and in the lack of specificity in the final products. We report here a novel approach for the fixation of CO₂ which combines a semiconductor photoelectrode (p-type indium phosphide, p-InP) with a biological catalyst (a formate dehydrogenase enzyme) to effect the first step, a two-electron reduction of CO₂ to formic acid. Photogenerated electrons in the semiconductor, which can be coupled to the enzyme through a mediator, can be produced with light of wavelengths shorter than 900 nm (>1.35 eV) so that much of the energy of the solar spectrum could be captured for this process. The process is analogous to natural photosynthesis but has the potential to be more efficient at light collection and more specific in the production of reduced carbon species.

Photoelectrochemical systems have been developed which show respectable solar efficiencies for the production of electrical power^{1,2}, for the generation of hydrogen from water³⁻⁵, and for the photoelectrolysis of haloacids⁶. The attempts at the more complex photoreduction of carbon dioxide to easily-stored liquid fuels have generally been less successful. Large band-gap semiconducting photoelectrodes or particles have been used⁷⁻⁹, but they absorb only a small fraction of the solar spectrum and thus have limited practical application. The large band gaps result in high-energy photogenerated carriers, needed to overcome the large activation energies associated with the first steps in the uncatalysed reduction of CO₂. These high-energy carriers tend to produce radical intermediates which, in the absence of specific catalysts, lead to a variety of reduced products (that is, mixtures of oxalate, formate, formaldehyde, methanol and methane)⁷⁻⁹.

The semiconductor InP has a band gap which is nearly ideal for the efficient conversion of solar photons to electrical or chemical energy. Recent work has demonstrated that stable and efficient photocathodes can be constructed from this material^{3-6,10}. Mott-Schottky measurements indicate that the conduction band energy of InP in aqueous solutions, with pHs between 6 and 8 is more negative than the thermodynamic potentials for the stepwise, two-electron reductions of CO₂. The photogenerated conduction band electrons also have the potential to produce hydrogen gas from water in the presence of noble metal catalyst islands on the electrode surface³⁻⁶. In the absence of noble metal catalysts, other chemicals having similar redox potentials can be reduced in preference to protons¹⁰. Methyl viologen (MV) is such a chemical having a redox potential ($E_0 = -0.446$ V)—which is less than the energy of photogenerated electrons in the conduction band of InP. Thus, the open-circuit photovoltage for the reduction of the viologen to the cation radical can be large (see Fig. 1). We have measured open circuit photovoltages of up to 0.75 V for this process.

Methyl viologen has been shown to interact with many formate dehydrogenase (FDH) enzymes isolated from diverse biological sources¹¹. For many anaerobic microbes FDH functions as a part of an enzyme complex involved in the conversion of formate to H₂ and CO₂ or, in some cases, the reverse¹². The enzymes are freely reversible *in vitro* and the reaction involves little or no free energy change at pH 7 (that is, $K_{eq} \approx 1.0$) (ref. 13). Reduced methyl viologen, with a redox potential slightly more negative than $E^0(\text{H}^+/\text{H}_2)$ at pH=7, is able to substitute the reducing equivalents usually supplied by H₂, presumably at

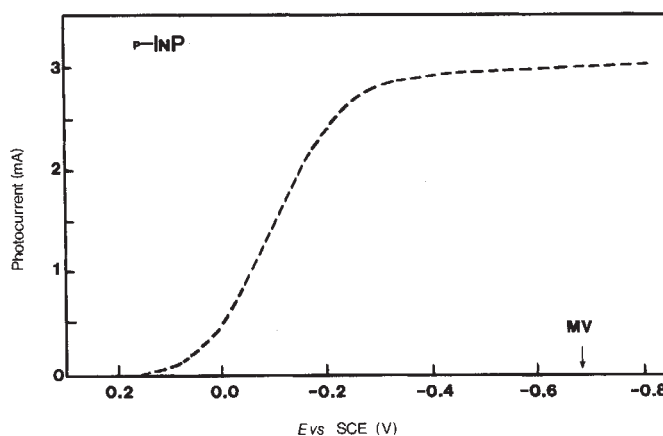


Fig. 1 Photocurrent-voltage curve for the cell p-InP/0.1 M MV²⁺, 1 mM MV⁺, 0.5 M potassium phosphate buffer (pH 6.8)/C under 50–60 mW cm⁻² illumination by a 150 W tungsten-halogen lamp. The p-InP doping level is 1.3×10^{18} cm⁻³ with Zn and the electrode area is 0.33 cm². The photovoltaic conversion efficiency of the cell is ~6–7%. MV indicates the methyl viologen redox potential compared with SCE (the standard calomel electrode).

the active site of the FDH enzyme. Although very little is known of the structure or molecular mechanism of FDH, several have been shown to contain combinations of non-haem iron, selenium, molybdenum and tungsten¹⁴, making them of interest to bioinorganic and biomimetic chemists.

We have successfully used photogenerated electrons from a p-type indium phosphide photocathode to reduce methyl viologen, which mediates the formate dehydrogenase-linked reduction of carbon dioxide to formate. Figure 2 shows a schematic energy diagram for our system. In one experiment, light from a 150 W tungsten-halogen source, incident on a p-InP photocathode potentiostated at +0.05 V compared with NHE (the normal hydrogen electrode) was used to generate blue coloured MV⁺ in one compartment of a two-compartment cell. Scrubbed carbon dioxide was continuously flushed through the magnetically-stirred solution in the working electrode cell compartment containing a 5 ml solution of a 0.5 M pH 6.8 phosphate buffer, 0.5 M sodium bicarbonate and 2 mM methyl viologen. After the injection of a solution containing 3 mg of a FDH preparation from *Pseudomonas oxalaticus* (Sigma), the blue colour disappeared within several minutes. Subsequent photogeneration of MV⁺ resulted in no build up of the blue colour in the solution even after the passage of 1.5 C of charge through the photoelectrode at about 0.6 mA cm⁻². Subsequent analysis of the solution for formic acid using the method of Wood and Gest¹⁵ revealed a formate concentration of 6×10^{-4} M resulting in a current efficiency of 80–93% for the synthesis of this product. The remaining reducing equivalents were most probably consumed by molecular oxygen generated at the counter electrode, located in the second cell compartment. Small quantities of O₂ presumably diffused through a glass frit separating the electrode compartments despite purging both sides with CO₂. No CO₂ reduction was observed if either MV²⁺ or FDH was omitted from the system.

The light flux used in this experiment was much higher than the mass transport flux of methyl viologen to the electrode surface. A relatively low MV²⁺ concentration was used because high concentrations, such as used in Fig. 1, interfere with the colorimetric formate analysis. This means that the light-to-chemical energy efficiency was low for this experiment; however, optimization of component concentrations, semiconductor properties and cell design could result in solar conversion efficiencies approaching those for H₂ generation from water (12%)³⁻⁵.

The 3 mg of protein preparation which was added to the reaction vessel contained ~1.5% active FDH based on the activity assayed by the manufacturer (0.65 μmol CO₂ from

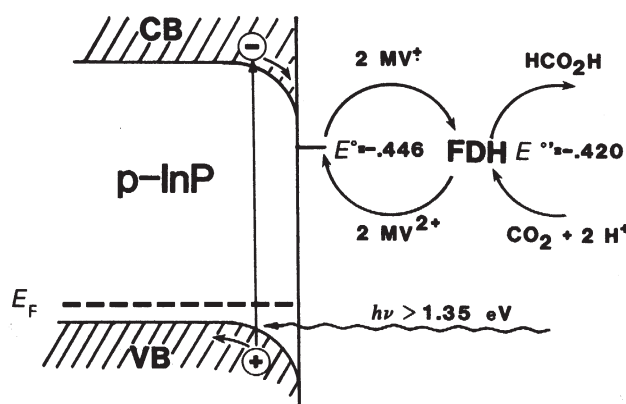


Fig. 2 The photoelectrochemical scheme for the photoreduction of CO_2 with the enzyme formate dehydrogenase (FDH) as the catalyst and methyl viologen (MV^{2+}) as the mediator.

formic acid per min per mg protein) and the maximum activity reported for a purified preparation of this enzyme ($42 \mu\text{mol CO}_2$ from formic acid per min per mg protein)¹⁶. The catalytic nature of the reduction was confirmed by calculating that the enzyme concentration was 23 nmol, resulting in a turnover number of 21,000 during the experiment (assuming a molecular weight for the enzyme of 320,000 (ref. 14)). The 1.5 C of charge passed also exceeds the total number of equivalents of methyl viologen in the system (1.2 C), indicating that the mediator was also recycled. The discovery of other mediators, especially inorganic species, would be useful because MV^+ is not thermally or photochemically stable for extremely long periods.

Electrochemical CO_2 reduction has suffered from the same deficiencies as the photoreduction. Large overpotentials and radical intermediates make the processes inefficient. A previous electrochemically driven system for CO_2 fixation used the multiple enzymes of green plant chloroplasts as catalysts¹⁷; this, however, required precursors and resulted in a low yield and a mixture of reduced carbon products. The more specific FDH-mediated reduction of CO_2 can also be accomplished electrochemically with minimal overpotential loss and a single product. Losses are minimized as the rate of electrochemical MV^{2+} reduction is fast and because of the small difference between the $\text{MV}^{2+}/\text{MV}^+$ redox potential and that of the $\text{CO}_2/\text{HCO}_2\text{H}$ couple.

Figure 3 shows the result of an experiment where a graphite foil electrode was used to electrolyse the methyl viologen mediator while formate concentration, produced by the action of FDH on CO_2 , was monitored as a function of time. The formate concentration levelled off after about 10–20 C were passed through the cell, which represents ~ 4 h; however, the current efficiency before the levelling off was close to that observed in the photoreduction experiment (89%). The initial low current efficiency could be the result of an induction period for the reaction or uncertainties associated with the formate analysis at the low concentrations. We interpret the cessation of formate production to the loss of enzyme activity due to denaturation of the protein. Formate dehydrogenases are typically quite unstable, especially towards O_2 . The vigorous stirring of the cell may have also contributed to denaturation of the enzyme. Immobilization of labile enzyme in support matrices or in whole cells greatly increases their stability¹³ and experiments of this kind are in progress.

Formic acid can be catalytically reconverted to carbon dioxide and hydrogen gas with very little loss of free energy. Several

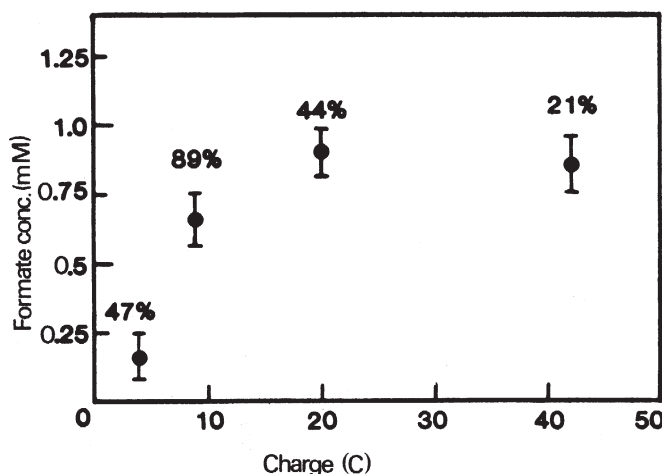


Fig. 3 Electrochemical reduction of CO_2 catalysed by formate dehydrogenase. A graphite foil electrode (area 57 cm^2) was used in one compartment of a two-compartment cell containing a 50 ml solution of 4 mg of enzyme preparation containing FDH, 0.5 M potassium phosphate buffer (pH 6.8), 0.3 M NaHCO_3 , and 1 mM MV^{2+} . Both cell compartments were purged with CO_2 and the working compartment was magnetically stirred. The numbers above the points indicate the approximate current efficiency for formate production after the passage of a given charge. Formate concentrations were determined from 0.5 ml samples anaerobically removed from the reaction at given intervals, quickly frozen in a dry ice/isopropanol bath, and subsequently analysed for formate using the method of Wood and Gest¹⁵.

investigators^{13,18,19} have proposed formic acid as a liquid form of chemically bound hydrogen, convenient for energy storage. The future possibility exists for an extension of the concept of coupling biological catalysts to photoelectrochemical energy collection systems by using additional specific enzymes capable of sequentially converting formic acid to formaldehyde and finally to methanol. Genetic engineering of microbes to enhance protein production could provide the quantities of enzymes needed.

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Tilman's predicted productivity–diversity relationship shown by desert rodents

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Tilman¹ has developed a model to predict the number of plant species that can coexist competitively on a limited resource base. Species diversity first increases over low resource supplies, then declines as the environment becomes richer. Although Tilman's model was developed to describe interspecific interactions between plant species, it may also apply to animal species. Tilman¹ questions whether animals specialize on particular proportions of nutrients. However, we believe animals probably specialize on relatively subtle microhabitat differences, especially in a multi-species competitive regime². Thus, microhabitats may act like nutrients. We hypothesize that animal species, too, show a peaked curve of diversity over productivity. The present data provide a confirmation of the hypothesis using rodent species. We have investigated the number of rodent species along a geographical gradient of increasing rainfall. The gradient extends from extremely poor desert habitats to those with annual rainfall over 300 mm. Because of the aridity, precipitation reflects productivity. The diversity pattern in desert rodents agrees with that predicted by Tilman for plants. It even possesses similar asymmetry, rising steeply then falling slowly. The pattern is duplicated in rocky and sandy habitats, each of which has a distinct and almost non-overlapping assemblage of species. As mean precipitation is closely correlated with the variability of precipitation, the diversity pattern might also be caused by a decline in the frequency of disturbances, models for which have been proposed by several investigators.

We worked in the sandy and rocky habitats of the Israeli arid zone. Sampling locations were chosen along a precipitation gradient. Rodents were sampled in four plots per location. Sampling was repeated for two seasons in the rocky habitat and four seasons in the sandy habitat.

The desert locations we studied occur on a steep gradient of annual rainfall (Fig. 1). Because in arid climates primary production is strongly correlated with rainfall³, we can use precipitation to represent productivity. Furthermore, because two different groups of rodent species inhabit the two habitat types studied (Table 1), we can test Tilman's model using two independent data sets.

Some 5,224 rodents were captured in the 13 locations of the sandy habitat. In the rocky habitat, 1,034 individuals were obtained in seven locations. We also used the seven rocky locations in the Sinai studied by Haim and Tchernov⁴ for which mean annual rainfall could be determined. In the rocky area, number of shrubs was estimated in each plot in 100 systematically selected 10-m transects. In the sandy area, annual cover was estimated in 12 systematically selected 10-m transects. We have averaged all estimates of the numbers of plants and rodent species to obtain estimates of mean conditions.

In both habitats, the peak in diversity of rodent species occurs in relatively poor locations and thus the curves have steeper ascending parts than descending parts (Fig. 1). If the precipitation axis is replaced by a productivity axis, the asymmetry is further exaggerated⁵. The two curves also have similar coefficients of regression (Fig. 1). In addition, the available data on rodents from sandy habitats of the deserts of the southwestern United States^{6,7} agree with the pattern found in Israel⁸, rising over the poorest habitats with about the same slope as in Israel. (Data for the downslope segment of this curve in the USA are not available.)

The existing literature contains conflicting evidence on the relationship between species diversity and productivity.

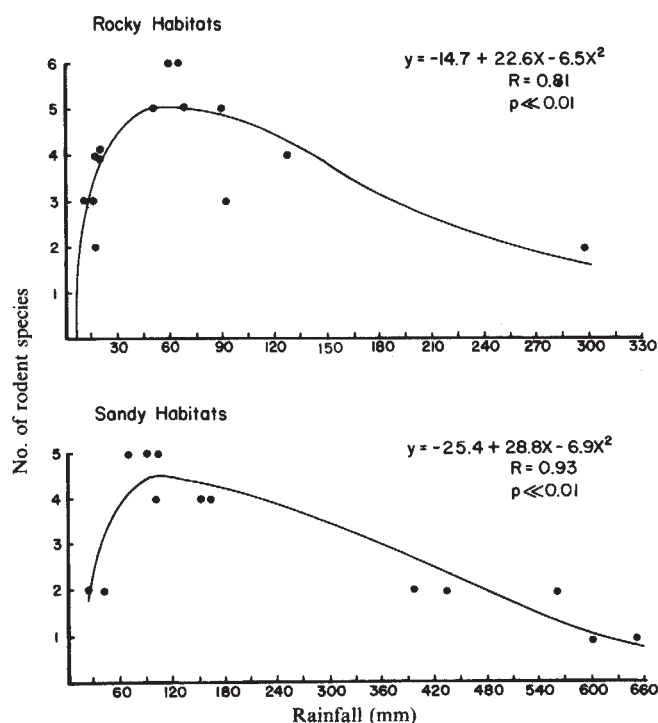


Fig. 1 The relationship between number of rodent species and rainfall in rocky and sandy habitats. The rainfall was log transformed for the regression analysis.

Table 1 Rodent species captured during the study

Species	Mean mass (g)	Sandy habitat	Rocky habitat
<i>Gerbillus gerbillus</i>	21.7	+	
<i>Gerbillus pyramidum</i>	39.9	+	
<i>Gerbillus allenbyi</i>	26.7	+	
<i>Gerbillus henleyi</i>	9.3	+	
<i>Gerbillus nanus</i>	23.0	+	
<i>Gerbillus dasyurus</i>	18.7		+
<i>Meriones tristrami</i>	78.8	+	
<i>Meriones sacramenti</i>	116.9	+	
<i>Meriones crassus</i>	53.3	+	+
<i>Acomys cahirinus</i>	37.8		+
<i>Acomys russatus</i>	42.5		+
<i>Sekeetamys calurus</i>	46.1		+
<i>Eliomys melanurus</i>	59.2		+

Theory⁹⁻¹¹, field studies^{6,7,12} and field experiments¹³⁻¹⁶ indicate both negative and positive correlations between productivity and species diversity. With the present study and Tilman's theory it is possible to reconcile these seemingly conflicting results: the direction of the correlation between productivity and diversity appears to depend on the position of a given study on the productivity gradient.

The increase phase of the relationship is relatively easy to understand. As resources become more abundant, additional species can subdivide the supply with each still acquiring a sufficient quantity to avoid extinction¹⁷. Why diversity declines in richer habitats is not so obvious, and several hypotheses have been suggested to explain it^{1,2,9,18-20}. None has yet been convincingly supported by data.

The decline of species number may merely reflect the increasing scarcity of some crucial part of the resource base, a resource which is declining even in the face of greater productivity. This supposition seems reasonable because rodent diversity and rodent-consuming biomass are closely correlated in both sandy habitats ($r = 0.86$, $P < 0.01$) and rocky habitats ($r = 0.67$, $P < 0.01$). Perhaps the supposition is true for the psammophilic

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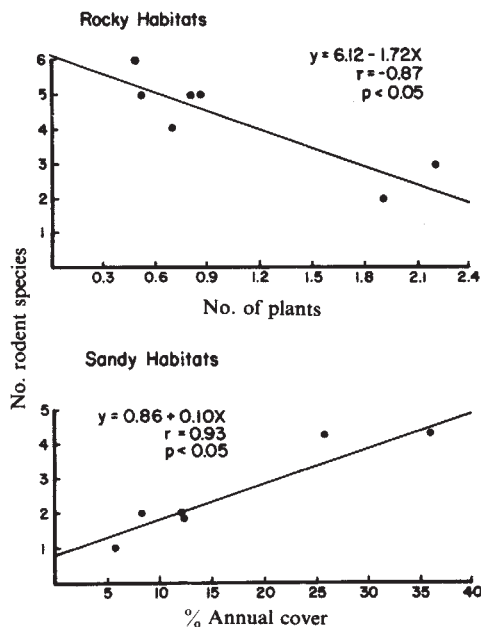


Fig. 2 The relationship between number of rodent species and a measure of productivity in rocky and sandy habitats. For both habitats, we selected only the locations from the declining part of the species diversity rainfall curve of Fig. 1.

rodents. These animals are primarily granivorous²¹, and both their diversity and biomass are positively correlated (Fig. 2) with per cent of annual cover (a good index of seed production). However, if there is a shortage, it is hard to say what it is for the omnivorous rock-dwelling rodent species because their diversity is negatively correlated with the total number of shrubs (index of overall production in this annual-free area). This is to be expected from Tilman's model.

Tilman's theory¹ is not the only one in which species diversity is a peaked function of an independent variable. A peaked curve is also obtained in habitats with different levels of disturbance, either physical²²⁻²⁶ or biological²⁷. Because the coefficient of variation of rainfall is inversely related to its mean²⁸, a gradient of disturbance occurs along our rainfall gradient. Perhaps it is this disturbance gradient which caused our results.

Although we have no data to distinguish between the productivity and disturbance hypotheses, nor to show whether they are actually the same as Tilman¹ suggested, our results do suggest that even in natural habitats, an enrichment may be followed by a decrease in species number.

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Magnetic orientation and magnetically sensitive material in a transequatorial migratory bird

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A variety of animal species is sensitive to changes in natural and artificial magnetic fields. The receptor mechanism for this ability has been described for a few species, most notably magnetotactic bacteria¹ and potential receptors have been reported for such animals as honey bees, homing pigeons and dolphins²⁻⁵. Some species of migratory birds also perceive changes in magnetic field⁶. We show here that the bobolink (*Dolichonyx oryzivorus*), which has the longest transequatorial migratory path of any New World land bird⁷, responds to changes in the Earth's magnetic field indicating that it uses the magnetic information as a primary orientation cue during its migration. We suggest that the ability of the bobolink to detect magnetic fields is associated with deposits of iron oxide (probably magnetite) that lie in sheaths of tissues around the olfactory nerve and bulb and between the eyes, and also in bristles which project into the nasal cavity.

The orientation of 14 wild-caught adults and 13 hand-reared birds was tested nightly in a planetarium. When celestial north and geomagnetic north coincided, both the wild-caught and hand-reared birds showed a preferred heading consistent with both cues (Fig. 1). When celestial north coincided with geomagnetic south, the birds reversed their preferred headings relative to the stars, but they maintained their headings relative to the geomagnetic field. Individual birds showed a lag of 2-5 days before changing their preferred headings relative to the stellar field when the relationship between the fields was altered⁸. This behaviour pattern is similar to that reported for the European robin⁹, but differs from that of the European warblers¹⁰ and most other North American species, such as the savannah sparrow^{11,12}.

The bobolink appears to use the geomagnetic field differently from the other avian species which show a sensitivity to magnetic fields^{13,14}. When the polarity of the total magnetic field was reversed by electrical coils, the preferred headings were altered⁹, indicating that the birds are able to discern either the direction of the total field or the horizontal component of the field.

The heads of 22 bobolinks (13 hand-reared birds and 9 wild-caught adults) were examined for magnetically sensitive material. The heads were fixed in 0.2 M phosphate-buffered glutaraldehyde (2.5%) and embedded in JB-4 plastic, or frozen (to measure remanence magnetization only). The natural remanence magnetization (NRM) of each head was measured using a Princeton Applied Research SM-2 magnetometer. The heads were then sequentially subjected to 1,000-, 2,000-, 3,000-, 4,000- and 7,000-Oe fields for 2 min and the isothermal remanence magnetization (IRM) measured after each exposure. The average NRM of the heads was $1.90 (0.45 \text{ s.d.}) \times 10^{-5}$ electromagnetic unit (e.m.u.) and the average saturation IRM was $4.17 (1.28 \text{ s.d.}) \times 10^{-5}$ e.m.u., similar to that reported for the homing pigeon⁴. The greatest gain in coercivity occurred

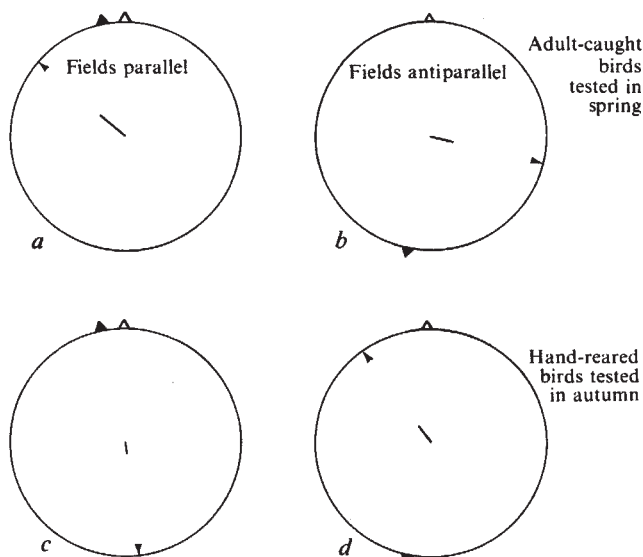


Fig. 1 Behavioural responses of bobolinks to visual and magnetic cues. *a*, Preferred headings of wild-captured adults in spring with the stellar and geomagnetic fields parallel ($\theta = 311^\circ$, $r = 0.3048$, $P < 0.001$, $n = 614$, $N = 21$). *b*, Preferred headings (relative to stellar north) of wild-captured adults in spring with the stellar and geomagnetic fields anti-parallel ($\theta = 105^\circ$, $r = 0.1949$, $P < 0.001$, $n = 334$, $N = 13$). *c*, Preferred headings of hand-reared birds in their first autumn migration period with the stellar and magnetic fields parallel ($\theta = 173^\circ$, $r = 0.0759$, $P = 0.05$, $n = 501$, $N = 24$). *d*, Preferred headings (relative to stellar north) of hand-reared birds in their first autumn migration period with the stellar and magnetic fields anti-parallel ($\theta = 333^\circ$, $r = 0.1358$, $P < 0.001$, $n = 695$, $N = 24$).

Methods: All tests were conducted in a planetarium with stationary star patterns projected onto the planetarium dome. Seasonally appropriate star patterns, which would be present at midnight, midway through the test season, were used. Wild-caught adults were tested nightly in the spring following their capture, and hand-reared birds were tested during their first autumn. The results of all tests for each condition were pooled as weighted vectors (first-order analysis). θ = angle of the mean vector, r = length of the mean vector, n = total activity based on an ordinal scale (0–20) developed by Emlen⁷, N = total number of bird-nights of activity, P = significance. Mean vectors and levels of significance were computed using circular statistics^{29–31}. The vector lengths (r) are proportional to the unit circle ($r = 1.00$). The arrow-head inside the circle periphery denotes the mean direction. Because first-order analysis was used, the significance levels are based on the total amount of activity for the pooled treatment (n). The open triangle indicates stellar north, and the solid triangle indicates magnetic north.

below 1,000 Oe, and most heads continued to gain remanence magnetization to 2,000 Oe (ref. 15).

All of the heads had attained maximum coercivity by 3,000 Oe. Multidomain magnetite has a maximum coercivity of 900 Oe (ref. 16), and any coercivity gain in magnetite above 1,000 Oe must be due to single-domain grains¹⁷. The theoretical maximum coercivity for single domain magnetite is 3,000 Oe, while other ferromagnetic materials continue to gain remanence magnetization above that level¹⁸. The remanence magnetization in the bobolink heads appears to be caused by a combination of single-domain and multidomain grains of magnetite. Although the domains in large multidomain grains of magnetite tend to cancel each other out, those in very small multidomain grains do not¹⁸. Such grains are termed pseudo-single domain grains and behave very similarly to single domain grains¹⁹. When individual heads were subdivided using glass and brass knives, the section from the posterior part of the nasal cavity to the anterior portion of the orbit had an average IRM of 5.0×10^{-5} e.m.u. ($N = 8$ birds), the same as in the intact head. The difference between the natural and inducible remanences may have been caused by a distortion of the tissues after death and

during fixation. In the living birds it is possible that the domains were aligned and that the NRM was equivalent to or near the IRM of our tests. Although the coercivity data and black colour of the iron oxide are not definitive, they are compatible with the hypothesis that most or all of the remanence magnetization in the bobolink is due to magnetite, the only known ferrimagnetic material of biogenic origin²⁰. Additional corroborative evidence from the Curie temperature and isotropic point, or definitive evidence from Mössbauer spectroscopy, would be desirable, but require the destruction of many birds. Regardless of the magnetic material involved, the observed IRM is large enough to allow sensitivity to even slight variations in the geomagnetic field^{21,22}.

Microscopic examination revealed that iron-rich material was consistently concentrated in sheaths around the olfactory nerve and in bristles which projected into the nasal cavity, as well as in a thin layer of tissue between the eyes near the olfactory bulbs (Fig. 2*a,b*). Magnetite has also been reported from these locations in the homing pigeon, but its presence was not consistent between or within individual birds^{4,23}. The heads were sectioned using glass knives, and the sections tested for iron using the prussian-blue reaction. The sheaths of tissue around the olfactory nerve and the bulb appeared to be composed of densely packed iron-oxide particles, probably the magnetic material detected with the magnetometer. Structures resembling bristle feathers which project into the posterior nasal cavities also responded to the prussian-blue test (Fig. 2*c,d*). These may respond to the magnetic field in a manner like a hair-cell mechanoreceptor²⁴. The location of this material becomes especially significant in light of experiments testing the importance of olfaction in pigeon homing²⁵. Many of those experiments, which cut, anaesthetize, or otherwise injure the area around the nares and olfactory tract, might also alter magnetic perception by the bird.

Areas responding to the prussian-blue test were also analysed using electron-probe X-ray analysis, scanning electron microscopy, and transmission electron microscopy. For transmission electron microscopy we relied on electron opaqueness to identify the iron granules. The electron-probe analysis used an ETEC autoprobe operating at 6.9 keV. Energy dispersive X-ray analysis was obtained using a KEVEX X-ray analyser. X-ray spectra were obtained in the STEM mode with a 10- μ m spot (30- and 300-s counts) and CTEM mode using approximately a 0.1- μ m spot (3-s counts). Energy dispersive X-ray analysis showed that the tissues which stained for iron contained large concentrations of iron (40–60 counts per s against a background of 2–5 counts per s in unstained but adjacent tissue), calcium and phosphorus; but no other metals such as nickel, chromium, cobalt or copper were detected. These results are similar to those reported for honey bees³ and chitons²⁶. The areas rich in iron appear to be associated with nervous tissue, based on scanning electron microscopy/X-ray analysis (Fig. 3). Viewing the re-embedded sections by transmission electron microscopy confirmed that nerves lie in close proximity to the iron-oxide particles. Because the iron oxide is adjacent to nerve tissue and it occurs in such high concentrations, we suggest that it is part of the bobolink's magnetic sensor.

In addition to the large concentrations of iron around the olfactory tissues, we found small deposits of iron scattered throughout the brain. This iron was yellow in colour, not the bluish-black of magnetite, and could be ferritin, which has been implicated in the production of magnetite in the chiton^{27,28}. When the electron beam was allowed to rest on the tissue in these areas, the iron quickly volatilized. The iron oxide-containing tissues showed neither volatilization of the iron nor change in the tissue.

This appears to be the first reported occurrence of magnetic material in a migratory bird which has been shown to respond to the Earth's magnetic field. Identification of all the magnetic materials in the bobolink is incomplete; however, at least some of it appears to be single domain and pseudo-single domain magnetite. The presence of nerve fibres in close proximity to

Fig. 2 Light (phase optics) micrographs of tissue sections containing iron-rich areas in bobolink heads. The sections were histochemically treated for the prussian-blue reaction (which is specific for iron) and counterstained with safranin O (to reveal cellular detail). Ot, olfactory tract; B, bone; I, iron oxide; Bf, bristle feathers. *a*, Section from around the olfactory nerve showing the sheath of iron oxide and its relationship to the other tissues, $\times 200$. *b*, Enlargement of a section from *A* showing the detailed structure of the iron oxide, $\times 500$. *c*, *d*, Bristle feather-like processes containing iron oxide which protrude into the nasal cavities, $\times 200$.

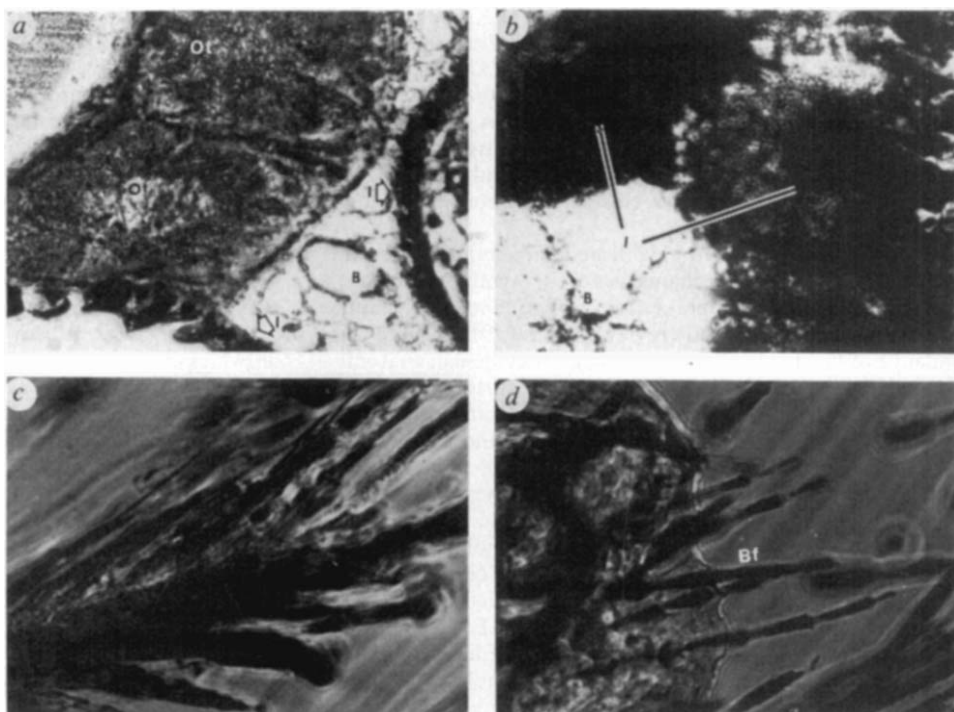


Fig. 3 Scanning electron micrograph of iron-containing tissue with X-ray fluorescence (seen as light points) electronically superimposed on the scanning electron micrograph image. Regions poor in iron show little or no X-ray fluorescence. The central thread-like structure appears to be nerve tissue (=N), $\times 1,000$, 2.7° tilt.

the iron oxide indicates that innervation of the tissue is likely. The mechanism of perception of the magnetic information by the bird is not yet known.

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Two types of cholinergic innervation in cortex, one co-localized with vasoactive intestinal polypeptide

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The existence of cholinergic neuronal cell bodies in mammalian cerebral cortex was long the subject of much controversy (see ref. 1 for review). Recently, however, a specific cholinergic marker, the acetylcholine synthesizing enzyme, choline acetyltransferase (ChAT, E.C.2.3.1.6), was demonstrated by immunohistochemical methods to be present in bipolar neurones in rat cortex^{2,3}. Here we show that at least 80% of these intrinsic cholinergic neurones also contain immunoreac-

tivity for vasoactive intestinal polypeptide (VIP), a neuroactive peptide found to be present in a subpopulation of cortical neurones⁴. On the other hand, we find that the ChAT-positive cells in the basal forebrain, which are another major source of cholinergic innervation of the cortex⁵⁻⁸, contain no detectable VIP-immunoreactivity. In addition, we have observed by both light and electron microscopy that some VIP- and some ChAT-positive structures in cortex are closely associated with blood vessels.

Acetylcholine is a well established neurotransmitter in cerebral cortex, where mechanisms for its synthesis, release, inactivation, high affinity uptake (for choline) and postsynaptic action are well characterized (reviewed in ref. 9). Despite this abundance of biochemical and physiological evidence, anatomical knowledge of the cholinergic circuitry in cortex is still preliminary. To expand our knowledge of this circuitry, in view of the remarkable similarity in the morphology and distribution of neurones containing either ChAT- or VIP-immunoreactivity², we have attempted to determine whether ChAT- and VIP-positive neurones are in fact the same cells.

ChAT and VIP were localized by immunohistochemical methods (ref. 10; see Fig. 1 legend for details) in cerebral cortex and basal forebrain of both normal and colchicine-treated (1mg per kg, intraventricular injection) Long-Evans rats. In agreement with previous findings²⁻⁴, both anti-ChAT and anti-VIP antisera stained neurones of similar morphology (Fig. 1*a, b*). Most of the neurones were bipolar in shape, having a fusiform cell body and two long, vertical, moderately branching processes. A few cells were observed to have more primary processes, up to a maximum of five. Measured on their short and long axis,

respectively, the ChAT-positive cells were $7.7 \pm 0.7 \times 13.1 \pm 1.4 \mu\text{m}$, and the VIP-positive cells were $7.6 \pm 0.7 \times 13.3 \pm 1.4 \mu\text{m}$ long ($\pm$ s.e., $n = 35$). The overall distribution of ChAT- and VIP-stained neurones in cortex was indistinguishable; labelled cells were present in all layers, but were most numerous layers II and III. Both antisera stained a few per cent of all cells in cortex.

To determine whether the two types of stained neurones were indeed identical, 6- μm serial cryostat sections from a small area of cortex, containing the most caudal part of the rhinal sulcus as a marker, were cut parallel to the surface of the cortex, mounted on gelatin-coated slides, and alternately stained for ChAT and VIP. Results were analysed by making camera lucida drawings of the stained sections, with blood vessels as landmarks. Figure 1*c, d* shows two neurones containing both antigens. Of 57 neurones stained for ChAT, 47 (82%) were found also to contain VIP in at least one of the two adjacent sections. This value for the percentage overlap may be too low, given the small size of these neurones and the difficulty in visualizing small fractions of cells that fall in adjacent sections. These results strongly suggest that ChAT- and VIP-immunoreactivity are contained in the same cells in rat cerebral cortex.

The ChAT-VIP neurones, however, do not represent the only system using acetylcholine as a transmitter in cortex. In fact, the cholinergic input from the basal forebrain appears to be the source of up to 70% of cortical ChAT^{5,6}. In contrast to the intrinsic cholinergic cells, however, the cholinergic cell bodies in the basal forebrain contained no detectable VIP-immunoreactivity, even after colchicine treatment (Fig. 1*e, f*). A recent report¹¹ indicates that a third type of cholinergic innervation

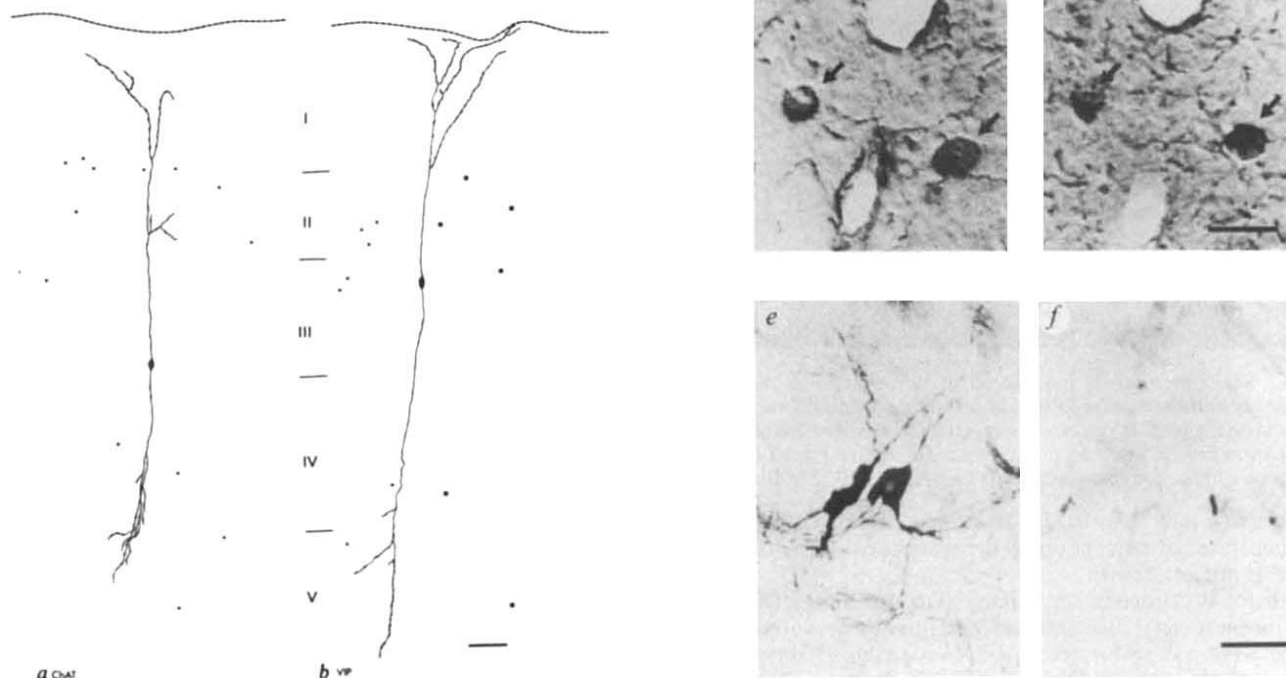


Fig. 1 Comparison of ChAT (*a, c, e*) and VIP (*b, d, f*) staining in rat cerebral cortex (*a-d*) and basal forebrain (*e, f*). *a, b*, Camera lucida drawings of two cortical neurones containing ChAT-immunoreactivity (*a*) and VIP-immunoreactivity (*b*) (scale bar, 40 μm). Both neurones are bipolar in shape with their cell bodies in layer III, with the ascending dendrite branching in layer I and the descending dendrite branching in layer V. *c, d*, Photomicrographs of two 6- μm serial sections stained for ChAT (*c*) and VIP (*d*) (scale bar, 10 μm). Two neurones (arrows), identified by alignment of blood vessels, contain both antigens. *e, f*, Photomicrographs of two 20- μm frozen sections from the nucleus basalis, stained for ChAT (*e*) and VIP (*f*) (scale bar, 20 μm). Throughout the basal forebrain (including the basalis) numerous large, multipolar, heavily ChAT-positive neurones are found. No VIP-immunoreactivity, however, is observed in these neurones, even after colchicine treatment. **Methods:** ChAT and VIP were localized in aldehyde perfusion-fixed frozen sections by sequential incubation in either anti-ChAT or anti-VIP antiserum (diluted 1:500 or 1:1,000, respectively), followed by biotinylated second antibody (diluted 1:100) and horseradish peroxidase coupled to avidin (diluted 1:500), then visualized with diaminobenzidine (see ref. 10 for characterization of antibodies and for further experimental details). Both an anti-ChAT antiserum and a monoclonal antibody to ChAT labelled cells of identical morphology and distribution, supporting the interpretation that the antigenic site being visualized is indeed ChAT. Preabsorption of the anti-VIP antiserum with synthetic porcine VIP abolished all specific staining. Preabsorption of the anti-ChAT antibodies with VIP, however, did not interfere with the staining.

of cerebral cortex, a projection from the midbrain and pontine tegmentum, may have substance P co-localized within it. This suggests that VIP and substance P may be useful as markers to differentiate between the various types of ChAT-positive terminals in cortex.

It is clearly of interest to determine the physiological role of the ChAT-VIP cells. In the present experiments we have observed with both the light microscope (Fig. 2a,b) and the electron microscope (Fig. 2c,d) that fibres and varicosities containing VIP- or ChAT-immunoreactivity are in intimate association with blood vessels. Both capillaries and larger blood vessels are involved. Our present data do not distinguish whether the ChAT-positive structures originate only from intrinsic ChAT-VIP cells or whether some are derived from cholinergic basal forebrain projections. The observed localization does suggest that acetylcholine and VIP may be released onto blood vessels and thereby affect blood flow, permeability or both. Previous studies have shown that VIP dilates cerebral arteries^{12,13}, besides having other effects in cortex¹⁴⁻¹⁷; that microvessels prepared from cortex contain specific VIP¹⁸ and muscarinic binding sites¹⁹; and it has been suggested that VIP may enhance muscarinic binding affinity in the submandibular gland²⁰. Taken together with our immunohistochemical findings, these results suggest that the ChAT-VIP neurones may be involved in regulating cortical blood flow. Many ChAT- and VIP-labelled structures were associated with neuronal profiles, however, suggesting that the ChAT-VIP neurones may have other functions as well. Interestingly, bipolar cortical neurones containing cholecystokinin-immunoreactivity also seem to be associated with blood vessels²¹.

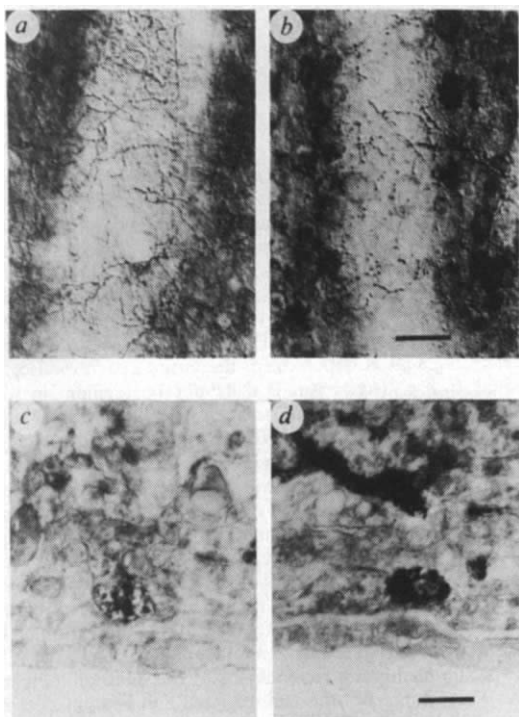


Fig. 2 ChAT(a,c)- and VIP(b,d)-containing structures in close association with cortical blood vessels. a, b, Photomicrographs showing evidence for the proximity of ChAT(a)- and VIP(b)-containing varicosities to the lumen of blood vessels (scale bar, 30 μ m). The lumen is the lighter area in the centre of the figure. c, d, Electron micrographs showing ChAT(c)- and VIP(d)-containing structures in close contact with cortical blood vessels (scale bar, 300 nm). The lumen is the clear area in the lower part of the figure. No clear evidence for synaptic specializations have so far been observed; this could be due to difficulties in preserving fine structure during the immunohistochemical procedure. (See ref. 22 for a similar method of immunoelectron microscopy.)

The targets of the other types of cholinergic innervation in cortex (the projections from the basal forebrain and the mid-brain) remain unknown. As indicated above, however, VIP and substance P appear to be differentially distributed within the ChAT-immunoreactive structures in cortex. This may provide a means of distinguishing between the different types of cortical cholinergic innervation, and repermit identification of their specific targets.

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Regional specialization of retinal glial cell membrane

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Neural activity generates increases in extracellular K^+ concentration, $[K^+]_o$, which must be regulated in order to maintain normal brain function¹. Glial cells are thought to play an important part in this regulation through the process of K^+ spatial buffering²⁻⁴: K^+ -mediated current flow through glial cells redistributes extracellular K^+ following localized $[K^+]_o$ increases. As is the case in other glia, the retinal Müller cell is permeable almost exclusively to K^+ (ref. 5). Recent experiments⁶⁻⁸ have suggested that this K^+ conductance may not be distributed uniformly over the cell surface. In the present study, two novel techniques have been used to assess the Müller cell K^+ conductance distribution. The results demonstrate that 94% of all membrane conductance lies in the endfoot process of the cell. This strikingly asymmetric distribution has important consequences for theories concerning K^+ buffering and should help to explain the generation of the electroretinogram.

Müller cell impedance was measured in enzymatically dissociated cells of the salamander (*Ambystoma tigrinum*), prepared as described in Fig. 1 legend. Cells were used within 2 h of isolation. Fine cellular processes, extending from the trunks of freshly dissociated cells, were resorbed within minutes of isolation and are not visible in Fig. 1. Depolarizing current pulses of 0.1-1.0 nA were passed through 3M potassium citrate pipettes (80-120 M Ω) and a bridge circuit, which was balanced before

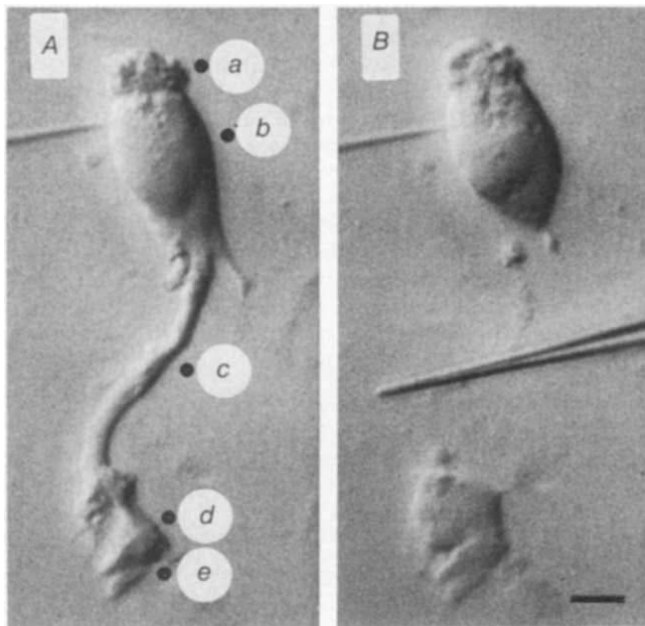


Fig. 1 Photomicrographs of a dissociated salamander Müller cell viewed with Nomarski optics. *A*, Intact cell penetrated in the nuclear region. Solid circles indicate locations of the K^+ -ejection pipette used in K^+ -ejection experiments. *a*, Distal end; *b*, nuclear region; *c*, stalk; *d*, lateral face of endfoot; *e*, proximal face of endfoot. *B*, Same cell after stalk has been cut by glass needle (shown in the centre of the photograph). Scale bar, 10 μ m. Dissociated cells were prepared by incubating isolated retinæ in Ringer's solution containing papain (Sigma P-3125; 0.75 mg ml $^{-1}$) for 30 min followed by gentle mechanical separation, as described previously¹⁶. The Ringer's contained (in mM): 82.5 NaCl, 27.5 NaHCO₃, 2.5 KCl, 1.8 CaCl₂, 1.0 MgCl₂, 10.0 dextrose, equilibrated with 5% CO₂ in O₂. Dissociated cells adhered to the bottom of the recording chamber, a glass slide coated with gelatin and concanavalin A (Sigma C-2010).

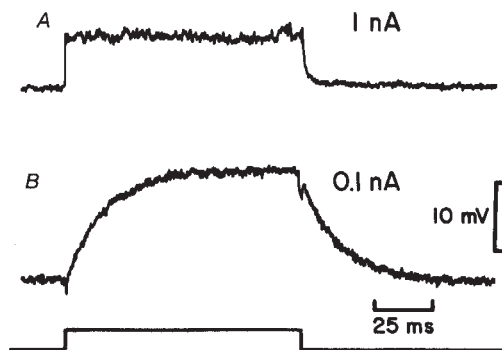


Fig. 2 Müller cell depolarizations evoked by current pulses applied through an intracellular pipette in the nuclear region of the cell. *A*, Response from an intact cell. Resistance = 7.4 M Ω ; τ = 0.86 ms (1 nA current pulse). *B*, Response from the same cell after the endfoot process was severed. Resistance = 156 M Ω ; τ = 16.7 ms (0.1 nA current pulse). The time course of the current pulse is indicated at the bottom.

cell penetration. Any residual voltage drop across the pipette (measured following electrode withdrawal) was subtracted electronically from intracellular records. Results are summarized in Table 1.

When Müller cells were penetrated near the nucleus (Fig. 1*A*) an average resistance of 8.8 M Ω and a membrane time constant (τ) of 0.95 ms were measured. If a large fraction of Müller cell conductance lies in the endfoot region, then cell

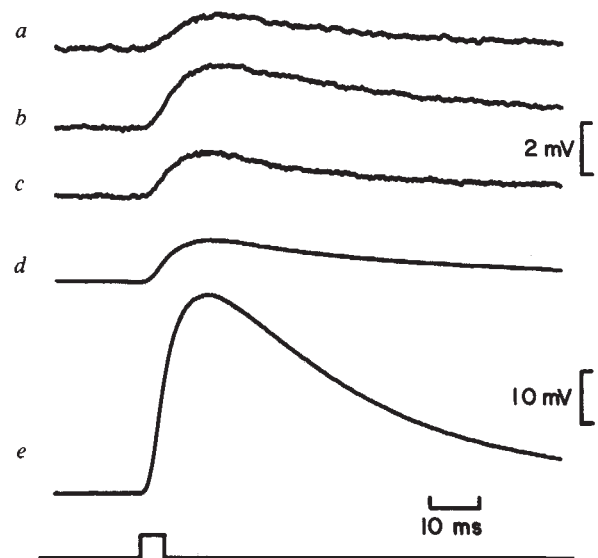


Fig. 3 Responses of a dissociated Müller cell (penetrated near the nucleus) to K^+ ejections from an extracellular pipette. The labels correspond to ejection locations, shown in Fig. 1*A*. Each trace is an average of eight sweeps. Onset and duration of a 5-ms pressure pulse is indicated at the bottom. Traces *a*–*c* are expanded vertically fivefold relative to traces *d* and *e*.

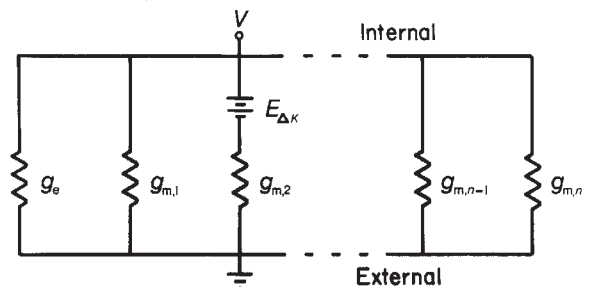


Fig. 4 Ohmic model of Müller cell simulates K^+ ejection results. The idealized cell is divided into n membrane segments each having conductance g_m and one membrane segment having conductance g_e , representing the high conductance endfoot. If we assume that a K^+ ejection depolarizes a single membrane segment, the amplitude of a K^+ ejection response can be calculated by interposing a voltage source in series with that membrane segment. This depolarization is represented by $E_{\Delta K}$ in the model. If the endfoot membrane segment is depolarized, the entire cell response, V_e , will equal $E_{\Delta K} g_e / (ng_m + g_e)$. If a membrane segment in some other cell region is depolarized, the cell response, V_m , will equal $E_{\Delta K} g_m / (ng_m + g_e)$. The ratio of responses, V_e / V_m , equals g_e / g_m . If more than one membrane segment is depolarized by a K^+ ejection, the ratio of responses will be reduced. If five segments are depolarized equally by a K^+ ejection, for example, the ratio of responses (endfoot to low conductance region) equals $(g_e + 4g_m) / 5g_m$, or roughly 1/5 of g_e / g_m when $g_e \gg g_m$. During experimental K^+ ejections directed towards the endfoot, an area significantly larger than the endfoot region was depolarized (unpublished observations). Consequently, the amplitude ratios of the responses shown in Fig. 3 lead to an underestimation of the value of the specific membrane conductance of the endfoot compared with the conductance of other cell regions. This analysis does not incorporate the effects of internal cell resistance on K^+ responses. Internal resistance will, in fact, reduce the amplitude of the endfoot K^+ response relative to the amplitudes of responses of cell segments nearer the recording site.

resistance should increase markedly if the endfoot process is removed. To test this, the stalk of individual cells was compressed with a glass needle (Fig. 1*B*) while the cell impedance was monitored. The membrane potential dropped to near zero as the stalk was cut and then, in many cases, repolarized as the cut stalk re-sealed. When membrane impedance was measured again in these endfoot-shorn cells, the mean cell resistance had increased 16-fold to 140 M Ω . The mean value of τ had increased

Table 1 Summary of impedance measurements in dissociated Müller cells

Recording site	Condition of cell	Resistance (M Ω)	Time constant (ms)	Capacitance (pF)
Nuclear region	Intact	8.8 $\pm$ 0.7 (55)	0.95 $\pm$ 0.07 (40)	149 $\pm$ 17 (40)
	Cut	140 $\pm$ 16 (30)	12.5 $\pm$ 1.8 (29)	94 $\pm$ 7 (29)
	Ratio cut/intact	15.9	13.2	0.63
Endfoot	Intact	12.5 $\pm$ 2.5 (14)		
	Cut	15.8 $\pm$ 2.9 (8)		
	Ratio cut/intact	1.26		

Values $\pm$ s.e. are given. The sample size is indicated in parentheses. Capacitance values are calculated from the relation: capacitance = τ /resistance. Time constants were not measured for endfoot penetrations.

13-fold to 12.5 ms. The impedance records from one cell are shown in Fig. 2. In this example, cell resistance increased 21-fold and τ increased 19-fold following removal of the endfoot process. The 16-fold increase in the mean cell resistance following endfoot removal indicates that 94% of the total conductance of an average cell lies at the proximal end of the cell, in or near the endfoot region.

In a parallel series of experiments, dissociated Müller cells were penetrated in the endfoot process. When the distal portion of the cell was severed, the mean cell resistance increased by only 26%. This small increase demonstrates that cutting the stalk does not, in itself, lead to a large resistance increase.

The high conductance of the endfoot membrane was susceptible to damage produced by microelectrode penetrations. When cells were impaled in the endfoot, measured cell impedance often rose during the penetration, particularly when the endfoot membrane was stressed mechanically. No similar rise in impedance occurred when other regions of the cell were penetrated. This lability of endfoot conductance presumably accounts for the larger impedance measured in endfoot penetrations of intact cells compared with that measured in penetrations near the nucleus (see Table 1). In addition, the conductance instability probably accounts for most of the 26% rise in impedance measured in endfoot penetrations when the distal portion of the cell was severed; following stalk bisection the isolated endfoot often appeared distorted (see Fig. 1B).

A second series of experiments was done to localize the region of high membrane conductance more accurately. Responses to local increases in $[K^+]_o$ were recorded from dissociated cells that were penetrated near the nucleus. $[K^+]_o$ increases were generated by pressure-ejecting an 85 mM KCl-Ringer's solution from an extracellular pipette positioned within a few micrometres of the cell surface. Results from one series of K^+ ejections are shown in Fig. 3. A K^+ ejection directed at the proximal surface of the endfoot produced a response (Fig. 3e) whose peak amplitude was 16–29 times greater than responses evoked by ejections directed towards other regions of the cell (Fig. 3a–c). Furthermore, when the ejection pipette was moved only 11 μ m, from the proximal (e) to the lateral (d) surface of the endfoot, the K^+ response amplitude dropped by 79%.

Mean values of the peak amplitude of the K^+ response (with the proximal endfoot response normalized to 100%) are as follows ($n=10$): lateral endfoot surface (d), 26.3 $\pm$ 3.7% ($\pm$ s.e.); stalk (c) 5.7 $\pm$ 1.0%; nuclear region (b), 4.6 $\pm$ 0.7%; distal

end (a), 4.0 $\pm$ 0.7%. Similar results were obtained from dissociated cells of frog (*Rana pipiens*) and turtle (*Pseudemys scripta*).

The amplitude of the response to a K^+ ejection is directly proportional to the conductance of that portion of the membrane experiencing the $[K^+]_o$ increase (assuming equal areas of membrane exposed; see Fig. 4). Thus, the K^+ ejection results confirm that Müller cell conductance is significantly greater in the endfoot process than in other regions of the cell. The results indicate further that the high membrane conductance is restricted largely to that portion of the endfoot surface facing the vitreous humour.

In summary, the results demonstrate that 94% of total Müller cell conductance is located within or adjacent to the proximally facing surface of the endfoot process. It appears that this membrane specialization will have an important effect on K^+ regulation in the retina, functioning as a key component of a Müller cell K^+ buffering system. Ninety-four per cent of all K^+ flowing into Müller cells from regions of increased $[K^+]_o$ within the retina will exit through the endfoot process, directly into the vitreous humour. The vitreous, in turn, will function as a nearly infinite K^+ sink. K^+ will accumulate there until retinal $[K^+]_o$ returns to normal. The spatial buffering system will then work in reverse, returning K^+ to the retina from the vitreous.

Müller cells are believed to generate several components of the electroretinogram (ERG)^{9–13}. High endfoot conductance will have an important effect on the magnitude of these potentials. Theoretical calculations¹³ show, for instance, that the Müller cell produces an ERG b-wave potential five times larger than would be generated if K^+ conductance were distributed uniformly throughout the cell.

Astrocytic glia in the central nervous system possess endfeet that lie adjacent to capillaries and the surface of the brain. If astrocyte endfeet have high K^+ conductance as do Müller cells (a type of astrocyte) then K^+ spatial buffering by astrocytes^{2–4} would be enhanced. High astrocytic endfoot conductance would direct excess extracellular K^+ directly into capillaries and the cerebrospinal fluid. The magnitude and orientation of field potentials generated by these cells^{14,15}, including components of the electroencephalogram, would also be altered by high endfoot conductance.

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Role for microsomal Ca storage in mammalian neurones?

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Alterations in the intracellular concentration of calcium ions ($[Ca^{2+}]_i$) are increasingly being found to be associated with regulatory functions in cells of all kinds. In muscle, an elevation of $[Ca^{2+}]_i$ is the final link in excitation-contraction coupling¹⁻³ while at nerve endings and in secretory cells, similar rises in $[Ca^{2+}]_i$ are thought to mediate exocytosis^{4,5}. The discovery of calcium-activated ion channels^{6,7} indicated a role for intracellular calcium in the regulation of membrane excitability. Calcium transients associated with either intracellular release or the inward movement of Ca^{2+} across the membrane have been recorded in molluscan neurones^{8,9} and more recently in neurones of bullfrog sympathetic ganglia¹⁰. Here, we report the first recordings of calcium transients in single mammalian neurones. In these experiments we have found that the methylxanthine, caffeine, causes the release of calcium from a labile intracellular store which can be refilled by Ca^{2+} entering the cell during action potentials.

Dorsal root ganglion (DRG) cells were dissociated from excised dorsal root ganglia of newborn rats and grown in cell culture according to methods similar to those previously described^{11,12}. The cells, which were attached to a Petriperm film (Heraeus), were mounted in a recording chamber on the stage of an inverted microscope and superfused with a balanced salt solution containing (mM) NaCl 130; KCl 3.5; $CaCl_2$ 5; HEPES 5; $MgSO_4$ 1; glucose 5; sucrose 40. All recordings were performed at room temperature (22–25 °C). In some experiments 20 mM tetraethylammonium (TEA) Cl was substituted for sucrose. Aequorin, 1–3 mg ml⁻¹ in 150 mM KCl and 5 mM HEPES buffer, was microinjected into a single neurone using changes of membrane potential and input resistance as indicators of the condition of the cell. Action potentials were elicited using current pulses 1–5 nA in amplitude for 1–10 ms. In some experiments, the aequorin-containing electrode was used both to pass current and to record membrane voltage. In others, current was passed through the electrode containing aequorin while accurate voltage recordings were made from a separate microelectrode filled with 150 mM KCl. Light was collected with a water immersion objective ($\times 50$, n.a. 1.0) and was recorded with an EMI 9789A photomultiplier tube cooled to around 0 °C.

In normal conditions it was not possible to record light associated with an increase of $[Ca^{2+}]_i$ during a single action potential. However, when a neurone was stimulated to fire a burst of action potentials (Fig. 1a) there was a slow increase in $[Ca^{2+}]_i$ which reached a maximum before the end of the burst and, after the burst, declined over a period of seconds. The increase presumably reflects a gradual accumulation of Ca^{2+} within the cell resulting from an influx of Ca^{2+} across the cell membrane, since it was completely blocked if 5 mM Co^{2+} was present in the extracellular solution. Using the technique of Allen and Blinks¹³ for the quantification of aequorin transients, we calculated that during this burst of action potentials $[Ca^{2+}]_i$ rose to a maximum of 3.5 μM . This value corresponds to an average increase in $[Ca^{2+}]_i$ of 18 nM per action potential and agrees

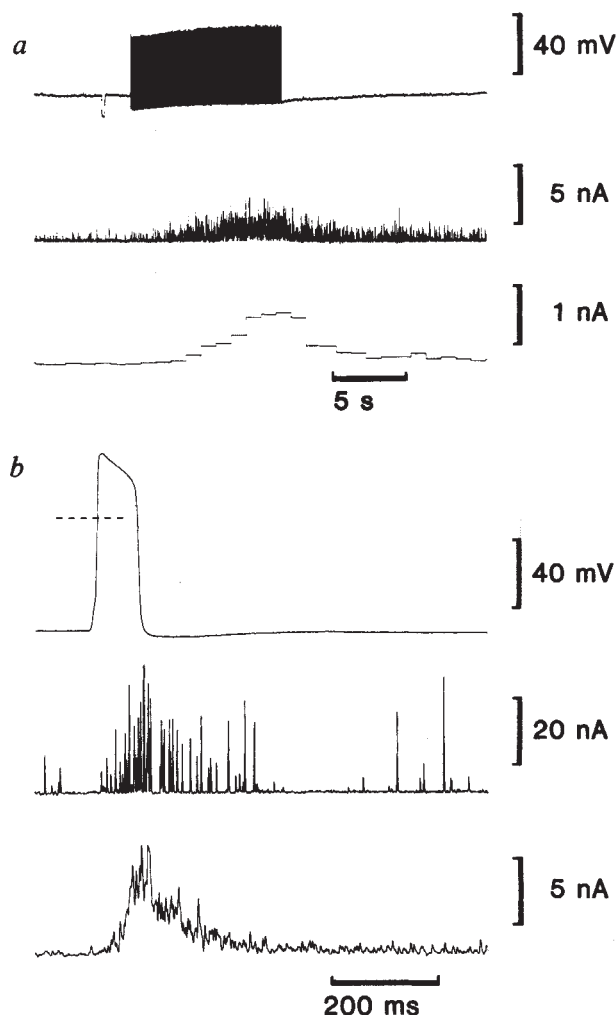
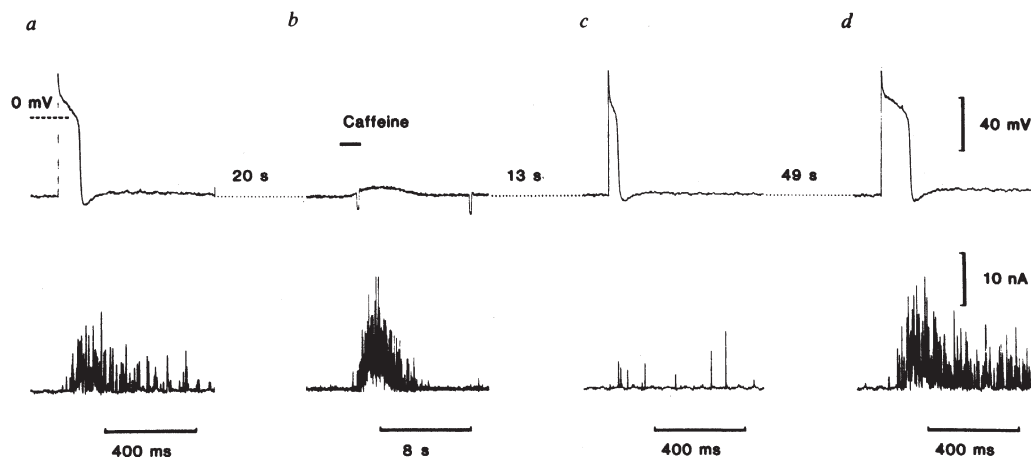


Fig. 1 Intracellular Ca^{2+} transients associated with electrical activation in single DRG neurones. *a*, The cell was stimulated at 20 Hz for 10 s by passing current through an intracellular electrode. The upper trace shows the voltage response (resting potential around -55 mV). Also shown on this record is the response to a hyperpolarizing current pulse 0.1 nA in amplitude used to monitor input resistance which was usually of the order of 100–200 M Ω for cells 30–35 μm in diameter. Action potentials have been 'clipped' in this record by the low-pass characteristics (d.c., 50 Hz) of the chart recorder. The middle trace shows the output from the photomultiplier used to record light from the aequorin-injected cell. Aequorin was purchased from Dr John Blinks, Mayo Foundation. The lower trace shows the signal from a device (R. N.McB. and I.R.N., in preparation) which generated a d.c. level corresponding to the value of the integrated light at 1-s intervals. This trace is much more conveniently quantified for long duration recordings of action potential bursts. Intracellular $[Ca^{2+}]_i$ was calculated using the technique of Allen and Blinks¹³. The fractional luminescence (L/L_{max} , where L is the light emission to be converted to $[Ca^{2+}]_i$ and L_{max} is the corrected peak light emission from the cell recorded at the end of an experiment on exposure of the aequorin to a saturating calcium concentration) was determined, applied to the appropriate *in vitro* calibration curve supplied with the aequorin and the corresponding $[Ca^{2+}]_i$ read off the curve. We assumed a $[Mg^{2+}]_i$ of 1 mM²⁸. *b*, The upper (voltage) trace shows an action potential obtained in another cell in the presence of 20 mM TEA. Resting potential was around -58 mV and the dotted line denotes 0 mV. The middle trace shows the simultaneously recorded output from the photomultiplier and indicates the elevation in $[Ca^{2+}]_i$ during the action potential. The light signal was filtered at 1 kHz. The lower trace shows a light response averaged from eight light transients associated with selected action potentials identical to the one shown. Responses were averaged on a Tracor Northern TN 1710 signal averager.

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Fig. 2 Effect of caffeine on $[Ca^{2+}]_i$ and electrical responses in a single DRG neurone. All responses were recorded in the presence of 20 mM TEA. *a*, Action potential (upper trace) and associated light transient (lower trace). Resting membrane potential was about -55 mV. *b*, Contiguous record 20 s after *a*. Note the prolonged time scale. Caffeine (10 mM) dissolved in the balanced salt solution containing 20 mM TEA was applied by the application of pressure (indicated by the heavy line) to a micropipette situated close to the cell. The peak $[Ca^{2+}]_i$ was around $10 \mu\text{M}$ and was associated with a small depolarization of the membrane potential. No action potential was elicited during this period. *c*, Contiguous record 13 s after *b*; the cell was again stimulated to fire an action potential; time was much shorter than previously and associated with much less light output. *d*, Contiguous record 49 s after *c* showing recovery of the action potential duration and associated light transient.



closely with the estimate obtained in neurones from bullfrog ganglia¹⁰.

Light associated with Ca^{2+} influx into DRG neurones during single action potentials could usually be detected if their duration was greater than about 20 ms (achieved by adding 20 mM TEA to the superfusate). A typical recording is shown in Fig. 1*b*. It can be seen that $[Ca^{2+}]_i$ rises slowly relative to the rising phase of the action potential and reaches a maximum at around the point of repolarization. This agrees with the idea that the aequorin signal is an integral of Ca^{2+} influx over the whole cell and that elevation of $[Ca^{2+}]_i$ activates a K^+ conductance which brings about repolarization. The correlation between the time courses of the after-hyperpolarizations and the declines of $[Ca^{2+}]_i$ (Fig. 1) confirms a relationship between $[Ca^{2+}]_i$ and hyperpolarization¹⁰. If one assumes spatial homogeneity of $[Ca^{2+}]_i$, then the peak concentration of Ca^{2+} reached during this action potential was $4.7 \mu\text{M}$ which corresponds to an increase of 66 nM ms^{-1} . This is about 1/50th of that which can be calculated from the voltage-clamp data of Dunlap and Fischbach¹² and suggests either that the current they attribute to Ca^{2+} is only partially carried by Ca^{2+} , which seems unlikely, or that buffering of free ionized calcium is taking place very rapidly within the cell¹⁴⁻¹⁶. Similar results were obtained in 12 other DRG neurones.

Dorsal root ganglion cells have a well-developed endoplasmic reticulum¹⁷. The possible role of this system in regulating $[Ca^{2+}]_i$ was examined using caffeine, which has been shown to release Ca^{2+} from the sarcoplasmic reticulum in muscle¹⁸ and also from microsomal fractions of muscle *in vitro*¹⁹. Figure 2 shows the effect of caffeine (10 mM) on $[Ca^{2+}]_i$ and membrane potential. Panel *a* shows a control action potential and the associated $[Ca^{2+}]_i$ transient. In Fig. 2*b* the application of caffeine caused a marked increase in $[Ca^{2+}]_i$ and a small depolarization of the membrane. Such depolarization was always seen when caffeine caused a detectable increase in $[Ca^{2+}]_i$ of cells showing an after-depolarization associated with an action potential. In those cells which did not show an after-depolarization, caffeine caused an increase in $[Ca^{2+}]_i$ and membrane hyperpolarization. Differences between the electrical responses of cultured DRG neurones to caffeine provides further evidence of the heterogeneity of neuronal cell types in dorsal root ganglia²⁰. Our observations agree with the suggestions that when caffeine causes depolarization, calcium may be activating nonspecific cation channels²¹ and, where caffeine causes a hyperpolarization, calcium is activating K^+ channels¹⁰. The source of calcium for this caffeine effect seems to be intracellular because in our experiments caffeine was able to cause an elevation of $[Ca^{2+}]_i$ when the extracellular solution contained 5 mM Co^{2+} . Figure 2*c* shows that an action potential elicited shortly after the caffeine-induced response has a shorter duration than the

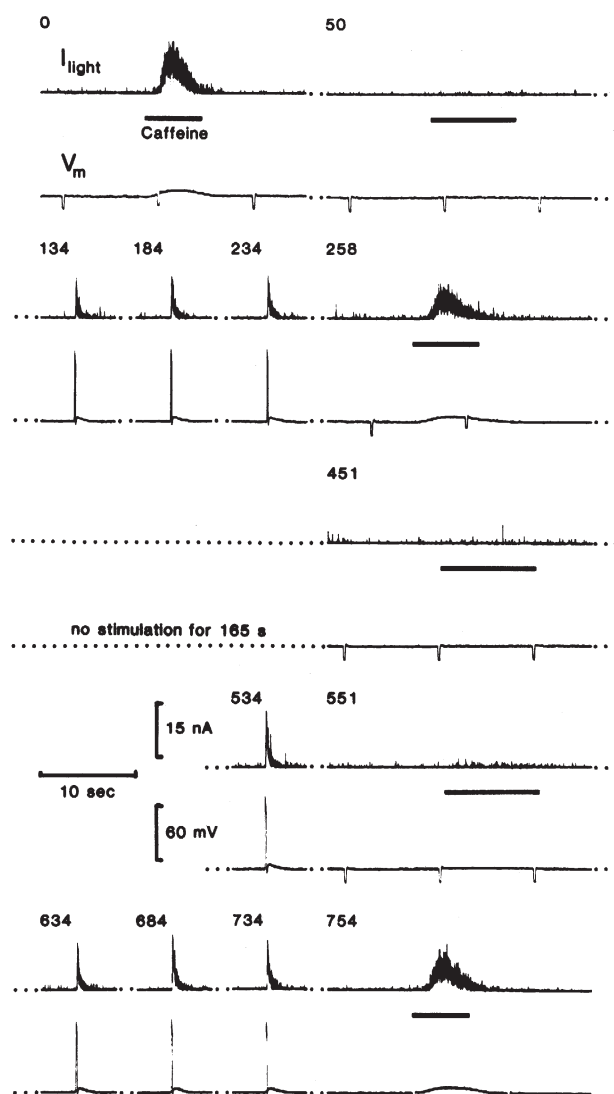


Fig. 3 Effect of electrical activation of a DRG neurone on the ability of caffeine to cause intracellular Ca^{2+} release. The figure is made up of a series of contiguous records of light output from the neurone (upper) and membrane voltage (lower) with time in seconds at the start of each trace indicated above the traces. Caffeine (10 mM) dissolved in balanced salt solution containing 20 mM TEA was applied as described for Fig. 2 during the periods indicated by the heavy black lines. See text for full description.

control (Fig. 2a) and is associated with a smaller Ca^{2+} transient. (Differences in the Ca^{2+} transients are exaggerated by the non-linear relationship between $[\text{Ca}^{2+}]$ and light output for aequorin.)

Possible explanations for this shortening of the action potential include: (1) indirect actions of caffeine, through inhibition of phosphodiesterase²², either to reduce the number of activatable calcium channels or to increase the calcium sensitivity of Ca^{2+} -activated K^+ channels²³; (2) a direct action of the elevated $[\text{Ca}^{2+}]_i$ to inactivate calcium channels^{24,25}. Although we have not directly tested any of these possibilities, in most experiments following the caffeine-induced rise, $[\text{Ca}^{2+}]_i$ fell to below the limit of detection ($\approx 5 \times 10^{-7}$ M) well before the action potential regained its control duration. Furthermore, in another series of experiments 0.1 mM isobutylmethyl xanthine (IBMX), a phosphodiesterase inhibitor²², caused a shortening of the action potential of DRG neurones without apparently increasing $[\text{Ca}^{2+}]_i$. Interestingly, in an unidentified neurone from the abdominal ganglion of *Aplysia*, IBMX prolongs the action potential²⁶. Since there is no evidence that IBMX releases Ca^{2+} from microsomes it seems that an inhibition of phosphodiesterase might underlie the effect of caffeine on action potential duration in these conditions. Figure 2d shows that the caffeine effect is reversible.

Figure 3 shows how the caffeine-sensitive intracellular store, once discharged, can be reprimed following electrical activation of the cell. The top traces show that, after an initial prolonged exposure of a neurone to 10 mM caffeine, a subsequent exposure causes negligible Ca^{2+} release from the store. Note also that when there is no release of calcium, there is no membrane depolarization or decrease in input resistance as previously observed. The next traces, contiguous with the last, show that following three action potentials, each associated with significant Ca^{2+} influx, caffeine once more causes an elevation of $[\text{Ca}^{2+}]_i$ and associated changes in membrane potential and input resistance. We suggest that caffeine releases Ca^{2+} from an intracellular store which can be replenished by Ca^{2+} moving into the cell during an action potential. That this repriming phenomenon is related to inwardly-moving Ca^{2+} associated with voltage activation and is not simply a function of time to allow equilibration of intracellular calcium stores or influx of Ca^{2+} through a 'leak', is shown in the next contiguous record. The preparation remained quiescent for a period of time similar to that over which the three previous action potentials had been elicited. Quite clearly, following this protocol, caffeine was unable to elicit any response. Caffeine was again tested after a single action potential had been elicited. There was a small but detectable increase in the output of light, indicating that a significant contribution of calcium to the intracellular store is made during a single action potential in these conditions. A further three action potentials once more returned the level of intracellular Ca^{2+} release by caffeine to around control levels.

The results described here indicate that there is rapid buffering of intracellular Ca^{2+} in rat DRG neurones. The calcium transient associated with an action potential declined with a time constant of 100–200 ms from a peak concentration considerably lower than that predicted from previously reported voltage-clamp experiments in chick DRG neurones¹². While the relative contributions of intracellular Ca^{2+} buffering, active pumping and sodium-calcium exchange to the maintenance of a low $[\text{Ca}^{2+}]_i$ remain unclear, there can be little doubt that these mechanisms could all play a part in the regulation of $[\text{Ca}^{2+}]_i$ (ref. 27). From the observation that caffeine causes a release of Ca^{2+} from intracellular stores and that these stores are refilled during electrical activity of the neurone, we suggest that the endoplasmic reticulum in mammalian neurones can have an important role in the control of $[\text{Ca}^{2+}]_i$ similar to the role of the sarcoplasmic reticulum in muscle.

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Conductances of single ion channels opened by nicotinic agonists are indistinguishable

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Hypotheses concerning the mechanism by which acetylcholine-like agonists cause ion channels to open often suppose that the receptor-ionophore complex can exist in either of two discrete conformations, open and shut^{1–3}. On the basis of noise analysis it has been reported that certain agonists open ion channels of lower conductance than usual^{4–8}, though many potent agonists give similar conductances^{9–13}, and hence that differences in the conductance of ion channels opened by different agonists may contribute to differences in efficacy¹⁴. Here we have reinvestigated this question by recording single ion channel currents¹⁵ evoked by acetylcholine-like agonists on embryonic rat muscle in tissue culture and on adult frog muscle endplate. Ten different agonists (Fig. 1) were tested, including several that noise analysis has suggested have a low conductance^{4,5}. The single-channel conductance was found to be the same, within a few per cent, for all 10 agonists. It seems that noise analysis has given erroneously low conductances in some cases. Therefore efficacy differences do not depend on differences in single-channel conductances evoked by various agonists but presumably on the position of the open-shut equilibrium of the agonist-channel complexes¹⁶.

The first set of experiments was carried out on dissociated embryonic rat muscle cells in primary tissue culture. Single ion channel currents were recorded by the patch-clamp method from cell-free patches in the 'outside-out' recording configuration, that is, with their external surface facing the bath solution¹⁵. Several agonists were applied to each outside-out patch. Records of single-channel currents for one outside-out patch, which had been sequentially activated by six agonists, are shown in Fig. 2a. The amplitudes of 30–50 unit currents were measured for each agonist at each of four or five membrane potentials, and histograms of amplitudes were plotted (Fig. 2b). In most cases the distributions were unimodal at each potential and could be fitted with a simple gaussian curve. However, some patches revealed a bimodal distribution of current amplitudes

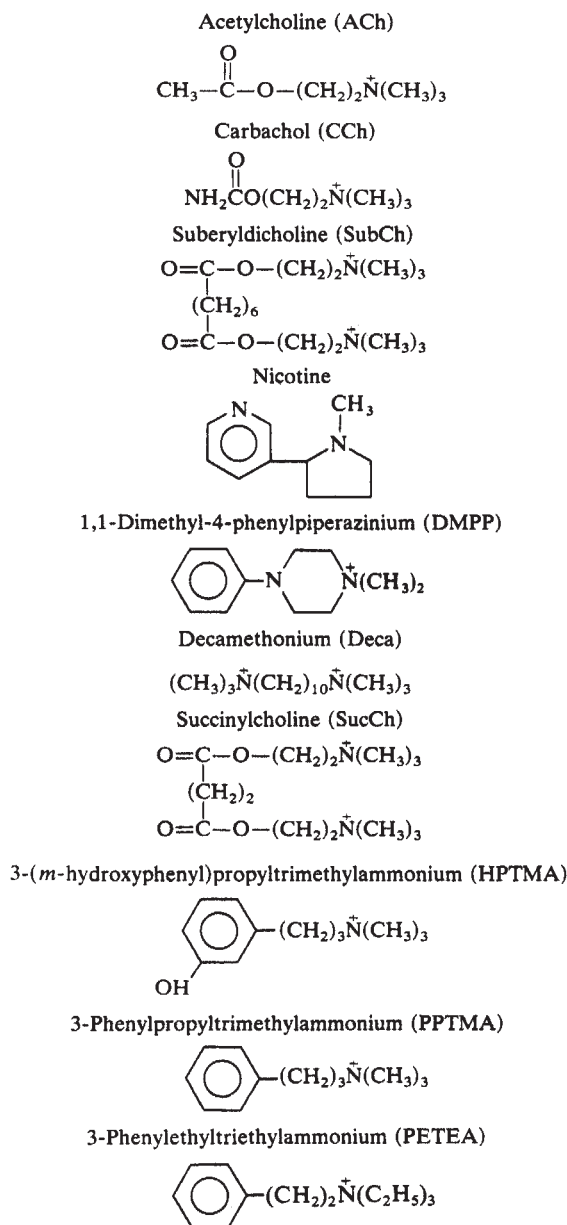


Fig. 1 Structures of the agonists tested.

at each potential; in one case where both conductances were measured, all six agonists tested elicited channels with the same two conductance values as those elicited by acetylcholine on the same patch. There was no evidence for preference by any agonist for one or the other channel type. Conductance sublevels of the sort reported in cultured embryonic rat muscle at 5–10 °C (ref. 17) and in cultures of chick myotubes¹⁸ were rarely seen in the present conditions. The current–voltage relationships for the agonists were determined by plotting the mean single-channel current amplitude as a function of patch potential. Figure 2c illustrates the current–voltage characteristics obtained for six different agonists applied to the same patch. The relationship is linear over the potential range tested and is very similar for each agonist. The slope conductance and reversal potential for each agonist were obtained from such plots. Extrapolated reversal potentials were close to 0 mV in all cases (mean $\pm$ s.e. = 2.78 ± 1.80 mV, $n = 26$). Single-channel slope conductance was quite variable; values of 30–55 pS were seen in different rat myotube patches, and sometimes more than one value was observed in the same patch. Because of this variability, acetylcholine was applied to every patch, and the results were expressed as the ratio of the conductance for each agonist to that for acetylcholine on the same patch (Table 1). The observed conductance ratios for nine agonists relative to acetylcholine

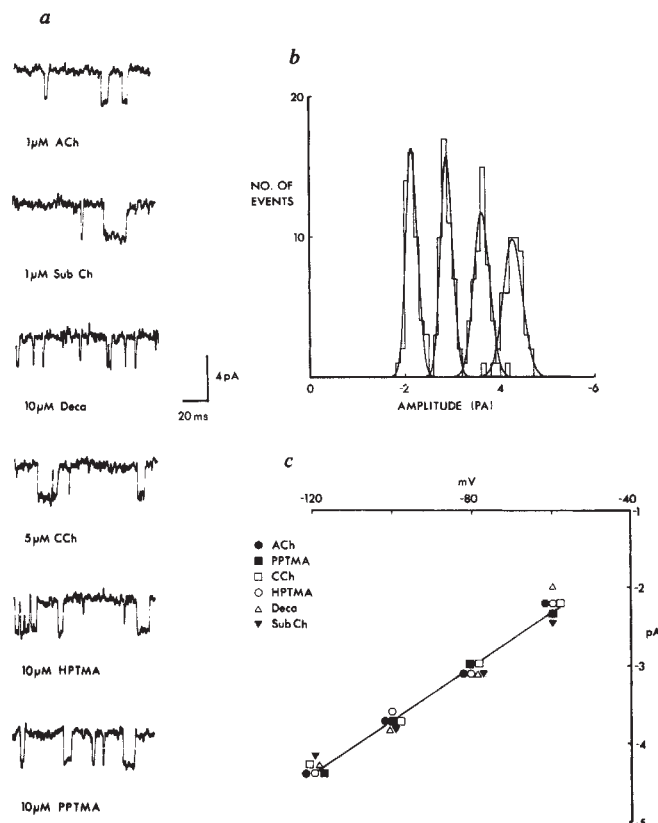


Fig. 2 Conductance properties of single acetylcholine-receptor channels activated by different cholinomimetic agonists. *a*, Single-channel current steps recorded from a cell-free, 'outside-out' patch isolated from rat myotubes. Membrane potential was -80 mV; temperature 20–23 °C. The recording pipette contained the following solution (in mM): KCl 150.0; Na₂EGTA 5.0; CaCl₂ 0.5; HEPES 10.0, pH 7.0. The bath solution was Eagle's minimal essential medium with HEPES buffer, at pH 7.2 (this contains 135 mM Na⁺ and 5.4 mM K⁺). Six different agonists were sequentially bath-applied as indicated under the individual recordings (ACh, 1 μM; CCh, 5 μM; SubCh, 1 μM; HPTMA, 10 μM; PPTMA, 10 μM; Deca, 10 μM). Application of each agonist was followed by washout during which disappearance of the single-channel currents was confirmed. A downward deflection denotes an inward current. All records are filtered with 2.0–3.0 kHz (-3 dB) low-pass Bessel filter. *b*, Distribution of current amplitudes at membrane patch potentials of -60, -80, -100 and -120 mV, during activation by 1 μM acetylcholine. The distribution at each potential is singly peaked with mean step sizes of -2.1 ± 0.1 pA at -60 mV, -2.9 ± 0.1 pA at -80 mV, -3.6 ± 0.2 pA at -100 mV, and -4.3 ± 0.2 pA at -120 mV. *c*, Current-voltage relation of single-channel currents from one outside-out patch that had been sequentially activated by ACh, CCh, SubCh, Deca, HPTMA and PPTMA. The mean amplitudes obtained from histograms as shown in *b* are plotted as a function of patch potential. The line represents the current-voltage curve obtained from least squares linear regression analysis of the mean current amplitudes and patch potentials obtained with acetylcholine. Similar curves were drawn for each agonist. The slope conductance was close to 34 pS for each agonist tested on this patch.

differ from unity by no more than 3%. These results provide no evidence to support the view that conductance of an ion channel depends on the nature of the agonist that causes it to open.

A second series of experiments was made at endplates of adult frog (*Rana temporaria*) cutaneous pectoris muscle fibres, after enzyme treatment¹⁹. Single-channel membrane currents were recorded with a patch pipette in the cell-attached configuration¹⁵, with the agonist in the pipette solution. Current–voltage relationships were constructed from unit current measurements at four or five membrane potentials over a 120-mV range, and slope conductances were determined, as described above. The slope conductances obtained with 10 different agonists for adult

Table 1 Single-channel slope conductance ratios for agonists relative to acetylcholine on rat myotubes

CCh (<i>n</i> = 3)	1.00 ± 0.03	SucCh (<i>n</i> = 2)	1.01 ± 0.04
SubCh (<i>n</i> = 3)	1.02 ± 0.05	HPTMA (<i>n</i> = 3)	1.00 ± 0.03
Nicotine (<i>n</i> = 2)	0.98 ± 0.02	PPTMA (<i>n</i> = 3)	1.01 ± 0.05
DMPP (<i>n</i> = 2)	1.00 ± 0.02	PETEA (<i>n</i> = 2)	0.98 ± 0.10
Deca (<i>n</i> = 3)	1.03 ± 0.05		

Values of the ratio of the single-channel conductance of the indicated agonist to that of acetylcholine on the same 'outside-out' membrane patch. Values represent mean ± s.e. (pS) with the number of determinations in parentheses. CCh, Carbachol; SubCh, suberyldicholine; DMPP, 1,1-dimethyl-4-phenylpiperazinium; Deca, decamethonium; SucCh, succinylcholine; HPTMA, 3-(*m*-hydroxyphenyl)propyltrimethylammonium; PPTMA, 3-phenylpropyltrimethylammonium; PETEA, 3-phenylethyltriethylammonium.

frog muscle endplate at 9–12 °C are given in Table 2. The actual conductances, rather than the values relative to acetylcholine, are given because conductance values were much less variable from one membrane patch to another on adult frog muscle endplate than on cultured embryonic rat muscle. The values obtained for each agonist fell within 5% of the weighted mean of all 10, 30.2 ± 0.09 pS (mean ± s.e., *n* = 10), a value similar to that found by Anderson and Stevens², but rather higher than most other estimates from noise analysis. Reversal potential was approximately 0 mV for all 10 agonists tested (mean ± s.e. = -0.2 ± 1.1 mV, *n* = 10). Note that each value given was obtained from a different muscle patch and that several muscles were used in this study.

We conclude that the single-channel conductances are the same, within a few per cent, for all 10 agonists tested. In particular this was observed to be the case for 3-(*m*-hydroxyphenyl)propyltrimethylammonium (HPTMA), 3-phenylpropyltrimethylammonium (PPTMA), nicotine and decamethonium, which had been reported on the basis of noise analysis^{4,5} to produce lower conductance channels than acetylcholine. The reversal potentials were also closely similar. This suggests that the open ion channel has essentially the same conformation whichever agonist opens it. Thus, it seems that agonist-receptor complexes formed by different agonists show differences in those regions of the macromolecules concerned with opening and shutting probabilities, but not in those regions concerned with ion permeation.

Recent observations have shown that the conventional concept of channel openings of fixed amplitude may be an oversimplification. It has been found that a single channel type may adopt unusual open states (sublevels), which have lower than normal conductance, in cultured rat¹⁷ and chick¹⁸ muscle, and occasionally in adult frog endplates²⁰. These discoveries suggest that differences in agonist efficacy could result from different degrees of preference for open-state sublevels of low conductance. This is unlikely, however, since conductance sublevels were rarely seen in this study with any of the 10 agonists.

It seems improbable that errors could cause apparent identity of single-channel currents that are in fact different, though it is possible that the absolute values from single-channel analysis could be erroneously high. Therefore, it appears that noise analysis has given erroneously low relative conductances for some agonists^{4,5}. Indeed a survey of the literature shows that noise analysis almost invariably gives lower estimates of the absolute single-channel conductance than those found from single-channel recording, though the size of the discrepancy varies from trivial to quite substantial. For example, acetylcholine-activated ion channels in rat endplate (diaphragm) have been reported to have conductances of about 30 pS (refs 21, 22) from noise studies, while channels of the rat endplate (omohyoid) have been reported to have conductances of approximately 58 pS (ref. 23) by single-channel recording techniques. Similarly it has been found that ion-channel blocking drugs appear to reduce single-channel conductance when tested by noise analysis^{24,25}, but no reduction is seen in single channel records (ref.

Table 2 Single-channel slope conductance on the adult frog muscle endplate

	γ (pS)		γ (pS)
ACh	30.0 ± 1.0	Deca	30.2 ± 0.1
CCh	29.8 ± 0.4	SucCh	30.0 ± 0.4
SubCh	29.9 ± 1.4	HPTMA	29.0 ± 0.4
Nicotine	31.4 ± 1.2	PPTMA	30.0 ± 0.3
DMPP	30.6 ± 1.2	PETEA	31.9 ± 0.4

Values of the slope conductance (γ) of channels activated by the indicated agonists on the frog muscle endplate. Values are expressed as slope conductance ± s.d. (pS). The recordings were made in the cell-attached configuration on frog muscle endplate at 9–12 °C. The bath and pipette solution contained the following (in mM): NaCl 116.5; KCl 2.5; CaCl₂ 1.5; HEPES buffer 10.0; tetrodotoxin 50 nM, at pH 7.2. Agonists were added to the pipette solution in the following concentrations: ACh, 100 nM; SubCh, 100 nM; CCh, 500 nM; nicotine, 500 nM; DMPP, 1 μ M; Deca, 1 μ M; SucCh, 500 nM; HPTMA, 500 nM; PPTMA, 1 μ M; PETEA, 10 μ M.

26 and D.C., D.C.O. and S. A. Siegelbaum, in preparation). This suggests that one or more of the assumptions that are used in noise analysis may be invalid. If all channels pass the same elementary current, i , then the channel conductance $\gamma = i/(V - V_{rev})$ is given by $\gamma = s^2/[m(V - V_{rev})(1 - p)]$ where m and s^2 are mean and variance of the agonist-induced current, V is the membrane potential, V_{rev} is the reversal potential, and p is the fraction of time for which a single channel is open. This expression is usually simplified by assuming that $p \ll 1$. It is valid regardless of whether or not channel block is occurring (as long as blockages are complete)^{24,25,27}. Thus the estimate of γ obtained from noise analysis would be too low (1) if the variance were underestimated (for example, by failing to extrapolate correctly the observed spectral components or by missing a spectral component altogether), (2) if the mean current, m , or the driving potential, $(V - V_{rev})$, were overestimated (for example, by imperfect voltage clamp of the active membrane), or (3) if the fraction of channels, p , opened by the agonist were too large to be neglected.

Thus, we have found closely similar single-channel conductances for 10 agonists. It appears that noise analysis may have underestimated the relative single-channel conductance in some circumstances, but it is not clear which assumptions of the noise analysis were breached.

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Atypical β -adrenoceptor on brown adipocytes as target for anti-obesity drugs

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Recent studies suggest that thermogenesis in brown adipose tissue has an important role in the regulation of energy balance¹⁻⁷. Thermogenesis is effected by noradrenaline released from sympathetic nerve endings; the noradrenaline stimulates β -adrenoceptors, causing lipolysis, and the released fatty acids then promote the uncoupling of oxidative phosphorylation from electron transport^{8,9}. It has been widely accepted¹⁰⁻¹³ that mammalian β -adrenoceptors exist as two subtypes, β_1 and β_2 (ref. 14), and rat brown adipocyte β -adrenoceptors have been classed as β_1 (refs 15-17) or as a mixed β_1/β_2 population¹⁸. The β_1 subtype predominates in atria, whereas the β_2 subtype predominates in trachea. However, we have now found a novel group of β -adrenoceptor agonists that selectively stimulate lipolysis in brown adipocytes. In contrast, isoprenaline, fenoterol and salbutamol are less potent as stimulants of lipolysis than as stimulants of atrial rate or tracheal relaxation. Therefore, β -adrenoceptors in rat brown adipocytes are of neither the β_1 nor β_2 subtypes. Compounds that selectively stimulate brown adipocyte β -adrenoceptors should have potential as thermogenic anti-obesity agents and this has been demonstrated with BRL 26830A, BRL 33725A and BRL 35135A.

The chemical structures of BRL 26830A, BRL 33725A and BRL 35135A are shown in Table 1. Following administration of BRL 26830A and BRL 33725A to rats, their free acids, BRL 28410 and BRL 35113, but not the parent compounds, could be detected in the plasma and it is believed that the free acids mediate their biological effects. It is also believed that the biological effects of BRL 35135A are mediated by its free acid, BRL 37344, because BRL 35135A is totally de-esterified within 15 min by human plasma and, following administration of BRL 35135A to rats, the acid but not the parent ester can be detected in urine (P. J. Baines, G. Mellows and A. J. Swaisland, unpublished data). Therefore, the free acids were used *in vitro* to study the nature of the β -adrenoceptor that mediates lipolysis in rat interscapular brown adipocytes. The effects of the free acids on rat right atrial rate (β_1 subtype) and guinea pig tracheal tension (mainly β_2 subtype) were also measured, for comparison with the effects on lipolysis. Precautions were taken to ensure that the experimental conditions met the criteria set out by Furchgott¹⁹ for the characterization of β -adrenoceptors (see Table 2). In particular, it was shown that the responses to the novel compounds were mediated by β -adrenoceptors alone, since they were competitively antagonized by propranolol (see Fig. 1 and ref. 20) but not affected by the α -adrenoceptor antagonists, phentolamine or phenoxybenzamine.

The concentration-response curves for the effect of the novel compounds on lipolysis (Fig. 1) and atrial rate (see ref. 20) were of a similar shape to the curves for the reference compounds (isoprenaline, fenoterol and salbutamol), except that the slope of the curve for BRL 35113 on atrial rate was low, consistent with the partial agonism of BRL 35113 for this effect. The slopes of the concentration-response curves for BRL 28410 and BRL 35113 on tracheal relaxation (see ref. 20) were low (two- and three-fold respectively less than for isoprenaline) but the slope of the curve for BRL 37344 was similar to that for the reference compounds (results not shown).

The relative potency of isoprenaline (non-selective between β_1 - and β_2 -adrenoceptors) for brown adipocyte lipolysis:atrial rate:tracheal relaxation was 1:7:4 (Table 2). The corresponding

Table 1 Chemical structures of novel β -adrenoceptor agonists

	R_1	R_2	Salt
BRL 26830A	H	CO ₂ CH ₃	$\frac{1}{2}$ Fumarate
BRL 33725A	CF ₃	CO ₂ CH ₃	HCl
BRL 35135A	Cl	OCH ₂ CO ₂ CH ₃	HBr
BRL 28410	H	CO ₂ H	—
BRL 35113	CF ₃	CO ₂ H	—
BRL 37344	Cl	OCH ₂ CO ₂ H	—

All the compounds are racemic mixtures in which both asymmetrical carbon atoms have the *R* configuration or both have the *S* configuration. This absolute stereochemistry was established by stereospecific synthesis of the enantiomers of BRL 28410 and confirmed by X-ray crystallography of BRL 26830A and BRL 35135A.

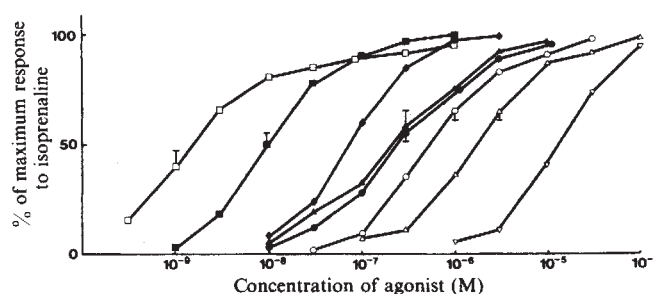


Fig. 1 Concentration-response curves for stimulation of lipolysis in brown adipocytes by isoprenaline (■), isoprenaline in the presence of propranolol (3×10^{-7} M) (◆), fenoterol (▲), salbutamol (●), BRL 28410 (△), BRL 28410 in the presence of propranolol (10^{-6} M) (▽), BRL 35113 (○) and BRL 37344 (□). Methods are described in Table 2 legend. The results for agonists alone are means of 4-10 values with bars for s.e.m. The results obtained in the presence of propranolol are means from two experiments.

relative potencies for fenoterol and salbutamol were 1:4.5:66 and 1:2.8:88, respectively. The selectivity of fenoterol and salbutamol for tracheal relaxation is consistent with their classification as selective β_2 -adrenoceptor agonists²¹, and suggests that the brown adipocyte β -adrenoceptor is different from the tracheal (β_2) adrenoceptor. However, the possibility that the atrial and adipocyte receptors are identical cannot be excluded by these results. Isoprenaline, fenoterol and salbutamol were 2.8-7-fold more potent as atrial than as lipolytic stimulants but this could be due to there being fewer β -adrenoceptors or weaker stimulus-response coupling in brown adipocytes than in atria^{19,22}.

If the β -adrenoceptors in brown adipose tissue and atria are identical, a lower number of β -adrenoceptors or weaker stimulus-response coupling in adipocytes would result in all β -adrenoceptor agonists being more potent stimulants of atrial rate than lipolysis. The results for BRL 28410, BRL 35113 and BRL 37344 show that this is not so (Table 2). BRL 28410, BRL 35113 and BRL 37344 were, respectively, 21-, 28- and 400-fold more potent as stimulants of brown adipocyte lipolysis than as stimulants of atrial rate. Indeed, BRL 37344 was 5-fold more potent than isoprenaline as a stimulant of lipolysis, but 523-fold less potent as a stimulant of atrial rate. Therefore, the brown adipocyte β -adrenoceptor cannot be of the β_1 subtype. The results for BRL 28410, BRL 35113 and BRL 37344 also strengthen the view that the brown adipocyte β -adrenoceptor cannot be of the β_2 subtype. These compounds were more potent as stimulants of lipolysis than as stimulants of tracheal relaxation, whereas the reverse was found for isoprenaline, fenoterol and salbutamol.

As there are β -adrenoceptor agonists that are selective stimulants of atrial rate and/or tracheal relaxation, and also a

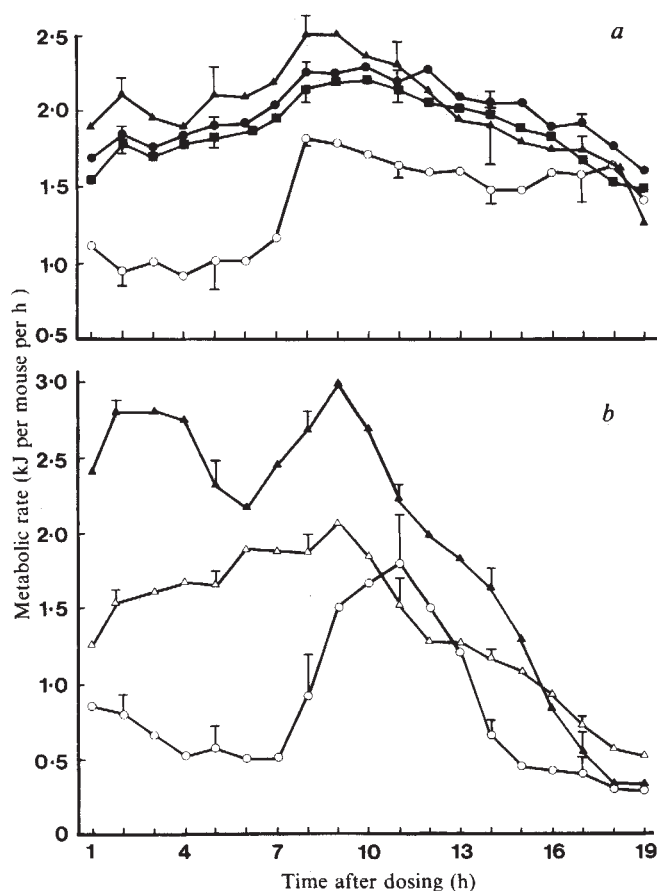


Fig. 2 *a*, Effects of single doses of BRL 26830A (▲, 10.3 mg (27.8 μ mol) per kg body weight, orally (p.o.)), BRL 33725A (●, 4.18 mg (10.0 μ mol) per kg body weight, p.o.), BRL 35135A (■, 0.64 mg (1.4 μ mol) per kg body weight, p.o.) and water (○) on the metabolic rate of female C57BL/6 (*ob/ob*) mice (mean body weight 30.0 g). The mice had been maintained at 26 °C in controlled lighting conditions (lights from 06.00 to 18.00 h). They were housed in pairs and transferred to a room at 23.5 °C for measurement of metabolic rate by open circuit indirect calorimetry²⁷. They were dosed at 11.45 h and allowed free access to food throughout the experiment. Results are means of three (○, ▲) or four (●, ■) values. Bars are shown for s.e.m. every third hour. *b*, Metabolic rate of genetically obese mice after their first (△) and 35th (▲) daily doses of BRL 26830A (0.2 mg per mouse, p.o.). The control mice (○) were dosed with water. Both the control mice and those receiving their first dose of BRL 26830A had been dosed with water on the 34 days before the experiment and their mean body weight was 45.4 g. The mean body weight of the mice that were receiving their 35th dose of BRL 26830A was 39.2 g. The mice were fasted from 09.00 h so that the thermic effect of feeding did not obscure the thermic effect of BRL 26830A (see ref. 27). Other details are described above.

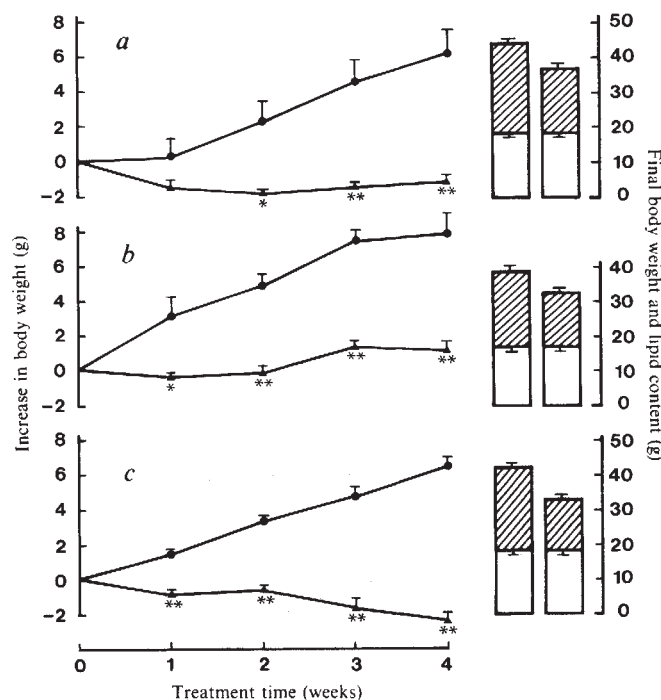


Fig. 3 Effect of daily treatment with BRL 26830A (10.3 mg per kg body weight, p.o.) (*a*), BRL 33725A (4.18 mg per kg body weight, p.o.) (*b*) and BRL 35135A (0.64 mg per kg body weight, p.o.) (*c*) on body weight gain and body lipid content of female C57BL/6 (*ob/ob*) mice. The mice were kept at 26 °C and dosed daily with compound (▲) or water (●) at 11.00–12.00 h. Their mean initial body weights were 38.3 g, 31.3 g, 35.9 g for *a*, *b* and *c*, respectively. Body composition was determined gravimetrically²⁷ after 4 weeks of drug administration. Body weight at this time is shown in the histograms, with body lipid content indicated by the hatched portion. Results are mean values, bars denote s.e.m., $n = 8$. * $P < 0.01$, ** $P < 0.001$, compared with control body weights. $P < 0.001$ for the effect of each compound on body lipid content.

inhibition by propranolol of the effects of isoprenaline and BRL 28410 on atrial and tracheal tension²⁰. Thus, propranolol has similar affinities for atrial and tracheal β -adrenoceptors, but 10–100-fold lower affinity for brown adipocyte adrenoceptors.

Selective β_1 - and β_2 -adrenoceptor antagonists also displayed a low affinity for the brown adipocyte β -adrenoceptor. The pA_2 value for atenolol (β_1 -selective) on BRL 37344-stimulated rat right atrial rate was 7.17 ± 0.04 and on guinea pig tracheal tension it was 5.65 ± 0.04 , whereas on lipolysis it was only 5.22 ± 0.13 . The pA_2 value for ICI 118, 551 (β_2 -selective) on BRL 37344-stimulated guinea pig tracheal relaxation was 8.72 ± 0.09 and on rat right atrial rate it was 7.19 ± 0.06 , whereas on lipolysis it was only 5.50 ± 0.08 . In each case the antagonist caused a parallel shift to the right in the dose–response curve for BRL 37344 and there was no significant regression of pA_2 on $\log[\text{antagonist}]$ ²³. Therefore, these results confirm the conclusion that the brown adipocyte β -adrenoceptor cannot be of the β_1 - or β_2 -subtype, nor a mixture of the two subtypes.

Since thermogenesis in brown adipocytes is stimulated by the fatty acids derived from lipolysis^{8,9}, β -adrenoceptor agonists that are selective stimulants of brown adipocyte lipolysis may be useful as anti-obesity drugs. This possibility has been investigated using BRL 26830A, BRL 33725A and BRL 35135A. These compounds caused large and sustained increases in metabolic rate in genetically obese (*ob/ob*) mice (Fig. 2*a*), consistent with the long terminal half lives of plasma radioactivity following administration of ¹⁴C-labelled BRL 26830A ($t_{1/2} = 9$ –13 h) or BRL 33725A ($t_{1/2} > 24$ h) to rats (P. J. Baines and G. Mellows, unpublished). Further studies revealed that the thermic effect of BRL 26830A was increased by repeat-dosing (Fig. 2*b*). The potentiation of the thermic response to BRL 26830A by 5 weeks of daily dosing was accompanied by a 5.5-fold increase in the

novel group of β -adrenoceptor agonists that are selective stimulants of brown adipocyte lipolysis, it follows that the brown adipocyte β -adrenoceptor cannot be of the β_1 or β_2 subtype, nor indeed a mixture of the two subtypes. This conclusion is supported by studies on the inhibition of lipolysis by β -adrenoceptor antagonists. Propranolol (10^{-7} , 3×10^{-7} , 10^{-6} and 3×10^{-6} M) caused parallel shifts to the right in the dose–response curves for isoprenaline and BRL 28410 (Fig. 1) and when pA_2 (negative logarithm of molar concentration of propranolol causing twofold shift in dose–response curves) was estimated from the shift caused by each concentration of propranolol, according to method (b) of Mackay²³, there was no significant regression of pA_2 on $\log[\text{propranolol}]$. However, the pA_2 values for inhibition of isoprenaline- and BRL 28410-stimulated lipolysis by propranolol were only 7.3 ± 0.2 and 6.8 ± 0.1 , respectively. These values were not significantly different ($P > 0.05$), but they are 1.0–1.9 units lower than the pA_2 values for

Table 2 Molar EC₅₀ values and relative intrinsic activities of β -adrenoceptor agonists

Agonist	Brown adipocyte lipolysis		Right atrial rate		Tracheal relaxation		Selectivity for brown adipocyte lipolysis over:	
	EC ₅₀	Relative intrinsic activity	EC ₅₀	Relative intrinsic activity	EC ₅₀	Relative intrinsic activity	Atria	Trachea
Isoprenaline	9.3 × 10 ⁻⁹ (6.0–14.4)	1.0	1.3 × 10 ⁻⁹ (1.2–1.4)	1.0	2.2 × 10 ⁻⁹ (1.2–4.0)	1.0	0.14	0.23
Fenoterol	2.2 × 10 ⁻⁷ (0.7–6.4)	1.0	4.8 × 10 ⁻⁸ (2.1–11.0)	1.0	3.3 × 10 ⁻⁹ (1.8–6.2)	1.0	0.21	0.02
Salbutamol	2.3 × 10 ⁻⁶ (1.1–4.9)	1.0	8.1 × 10 ⁻⁷ (3.3–20.0)	0.91 ± 0.03	2.6 × 10 ⁻⁸ (1.2–5.6)	1.0	0.35	0.01
BRL 28410	1.8 × 10 ⁻⁶ (1.2–2.8)	1.0	3.7 × 10 ⁻⁵ (2.2–5.9)	0.69 ± 0.04	1.5 × 10 ⁻⁵ (1.1–2.1)	1.0	20.6	8.3
BRL 35113	5.4 × 10 ⁻⁷ (2.8–10.0)	1.0	1.5 × 10 ⁻⁵ (1.0–2.2)	0.44 ± 0.00	1.8 × 10 ⁻⁵ (1.1–3.2)	0.92 ± 0.02	27.8	33.3
BRL 37344	1.7 × 10 ⁻⁹ (0.7–3.8)	1.0	6.8 × 10 ⁻⁷ (3.6–12.6)	0.80 ± 0.03	3.5 × 10 ⁻⁸ (1.2–10.5)	1.0	400	20.5

Glycerol release from rat interscapular brown adipocytes was measured⁸ over 30 min in the presence of various concentrations of the agonists. Each agonist concentration was used in triplicate within a single concentration–response curve. Bovine serum albumin (4%) was added to bind the fatty acids released. Preliminary experiments established that the time course of glycerol release was close to linear over 30 min and that reduction of the albumin concentration, whilst reducing the rate of lipolysis, did not significantly alter the relative potencies of the agonists. Thus, if the agonists bound to the albumin, this did not affect the interpretation of the results. Cumulative concentration–response curves for stimulation of rat isolated spontaneously beating right atrial rate and relaxation of the guinea pig intrinsic tone tracheal zigzag preparation were constructed according to the method of van Rossum³² as described previously²⁰. It was unnecessary to include inhibitors of catecholamine uptake in any of the experiments to ensure diffusion equilibrium between the bathing media and the region of the receptor, because in preliminary experiments 10 μ M cocaine (uptake₁ inhibitor) and 30 μ M phenoxybenzamine (preincubated with tissue and washed out) or 50 μ M metanephrine (uptake₂ inhibitors) did not potentiate any of the responses. However, tropolone (100 μ M) was included in the lipolysis experiments to prevent inactivation of isoprenaline by catechol-O-methyltransferase released from ruptured cells. In some preliminary experiments tropolone caused a slight (< twofold) decrease in the EC₅₀ value for isoprenaline. Molar EC₅₀ values are expressed relative to the agonist's own maximal effect as geometric means of 4–10 values with 95% confidence limits in parentheses (same exponent as the mean). Relative intrinsic activities are the maximal effect of the agonist/the maximal effect of isoprenaline and are expressed as arithmetic means of 4–10 values $\pm$ s.e.m. Relative intrinsic activities not significantly different from 1.0 are shown as 1.0 without s.e.m. Selectivity ratios are ratios of EC₅₀ values.

total mitochondrial protein content of interscapular brown adipose tissue and a 3.9-fold increase in atractyloside-insensitive binding of GDP per mg mitochondrial protein, so that there was a 22-fold increase in total GDP binding²⁴. The increase in GDP binding is believed to be due to the increased tissue content of the 32,000 molecular weight protein that permits uncoupling of oxidative phosphorylation in brown adipose tissue²⁵ and gel electrophoresis studies have shown that the total tissue content of this protein is greatly increased²⁴. These changes mimic those that occur during cold-acclimation²⁶ and are indicative of an increased responsiveness of the brown adipose tissue to β -adrenoceptor stimulation. This suggests that enhancement of the thermic effect of BRL 26830A was due to the increased thermogenic capacity of brown adipose tissue.

When genetically obese mice were dosed daily with BRL 26830A, BRL 33725A or BRL 35135A, the thermogenic activities of the compounds resulted in decreases in the rate of weight gain (Fig. 3). Although each compound reduced food intake during the first few days of treatment, this effect did not persist after the first week and total food intake over 4 weeks was not reduced by any of the compounds. Since the reduction in weight gain continued after the first week, it must have been due to increased energy expenditure. Analysis of body composition at the end of the experiments showed that the differences in body weight between the treated and control mice were entirely due to reductions in body lipid content (Fig. 3). There were no differences in water content or lipid-free dry weight between the control and treated mice. Similar results have been obtained in genetically obese (*fa/fa*) Zucker rats²⁷.

The present result is the first to show that β -adrenoceptors of brown adipocytes do not conform to the β_1/β_2 classification. Other studies^{28–31} indicate that β -adrenoceptors of rat epididymal white adipocytes also fail to conform to the β_1/β_2 classification. It therefore appears that, in the rat, brown and white adipocytes have similar, though not necessarily identical, β -adrenoceptors. If the β -adrenoceptor that mediates ther-

mogenesis in man also fails to conform to the β_1/β_2 classification, it should be feasible to develop selective thermogenic β -adrenoceptor agonists for the treatment of obesity.

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A novel human lymphokine that inhibits haematopoietic progenitor cell proliferation

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T lymphocytes in culture synthesize and secrete a variety of factors¹⁻⁶ that activate and guide the differentiation^{3,4}, replication⁵ and maturation⁵ of haematopoietic cells *in vitro*. Malignant T-cell lines as well as T-cell hybridomas producing several of these factors have been established⁵⁻¹³. We report here a factor produced by a human cell line that exerts a potent inhibitory effect on the growth of bone marrow progenitor cells. The properties of this factor, which we have termed colony-inhibiting lymphokine (CIL), differ from other inhibitors of haematopoietic progenitor cell proliferation, but resemble those of a T-cell-derived factor causally linked with some cases of severe aplastic anaemia in humans¹⁴. Sensitivity of cells to this factor appears to correlate positively with expression of HLA-DR surface antigens.

We isolated a 6-thioguanine-resistant mutant (6TM) of the Jurkat⁷ human T-lymphoma cell line after ethylmethane sulphonate mutagenesis and 6-thioguanine selection. Clone 6TM was fused with phytohaemagglutinin-stimulated peripheral blood lymphocytes from a healthy female volunteer using polyethylene glycol (PEG) 1500 (ref. 15). One of the seven hypoxanthine-azaserine-resistant¹⁶ colonies detected 2 weeks after fusion inhibited both spontaneous and induced colony formation of granulocytic-monocytic progenitor cells (CFU-GM) in an *in vitro* assay¹⁷. All clones derived from the primary colony retained the ability to secrete the factor. Subclone MT1 has produced the factor consistently for 11 months. The MT1 and 6TM supernatants, from large frozen stocks, were compared in culture assays to evaluate their effects on the growth of late (day 7, less immature CFU-GM), early (day 14, more immature CFU-GM) bone marrow progenitor cells¹⁸ and CFU-GM from peripheral blood leukocytes (Table 1). MT1 supernatant added to the culture medium at a final concentration of 5% completely inhibited the growth of late marrow CFU-GM, but only slightly inhibited growth when used at concentrations less than 1%. By contrast, MT1 supernatant as low as 0.001% inhibited early marrow CFU-GM colonies. All of the colonies that did form in day-14 cultures after treatment with MT1 supernatant at concentrations between 5 and 0.01% were identified morphologically and histochemically as macrophagic. Because myeloid colonies from peripheral blood leukocytes are detectable only after 8-10 days in culture, the growth of these colonies was scored 10 days after plating and was completely inhibited by the MT1 supernatant at a 0.01% final concentration. No macrophagic colonies were observed when peripheral blood leukocytes were plated. Control supernatant of the 6TM cell line failed to have any inhibitory effect, even at concentrations of 10% on colony formation from either bone marrow or peripheral blood leukocytes. Results of experiments using a constant concentration of MT1 supernatant and different dilutions of GCT-conditioned medium, as a source of colony stimulating factor (GCT-CM), indicated that the titre of MT1 supernatant did not change with GCT-CM dilutions, and CIL at a 0.01% concentration was still able to inhibit CFU-GM growth completely at all GCT-CM dilutions tested.

Day-14 CFU-GM from both peripheral blood and bone marrow are about 100 times more sensitive to the inhibitory effect of CIL than bone marrow day-7 CFU-GM. We have previously shown that circulating as well as a large proportion of day-14 bone marrow CFU-GM represent a separate and more immature

Table 1 Inhibitory effect of clone MT1 supernatant on CFU-GM growth

Culture in presence of Medium (control) MT1 supernatant	Bone marrow CFU-GM		Peripheral blood leukocytes CFU-GM
	Day 7 (late)	Day 14 (early)	Day 10 (early)
	100	100	100
10%	0	0	0
5%	6 ± 7	4 ± 9	0
1%	34 ± 52	6 ± 9	0
0.1%	77 ± 8	10 ± 4	0
0.01%	91 ± 1	29 ± 2	0
0.001%	—	48 ± 5	10 ± 6
0.0001%	—	93 ± 4	90 ± 5
6TM supernatant			
10%	101 ± 4	108 ± 8	114 ± 10

Bone marrow and peripheral blood myeloid progenitor cells (CFU-GM) were grown in semisolid medium using a modification of the technique described by Iscove *et al.*¹⁷. Haematopoietic low-density (<1.077 mg ml⁻¹) non-adherent cells from normal donors were cultured in 35-mm Petri dishes in Iscove's modified Dulbecco's medium containing 20% fetal calf serum, 0.3% agar, 10% GCT-conditioned medium⁹ (Gibco) as a source of CSF and different concentrations of clone MT1 supernatant. MT1 and 6TM cells were cultured in RPMI 1640 medium containing 10% fetal calf serum, 100 µg streptomycin, 100 units penicillin, and 2 mM glutamine. The same conditions (5 × 10⁵ cells per ml and collection 3 days after last feeding) were used to prepare both stocks of supernatant. MT1 cells cultured in 'synthetic' medium (RPMI 1640, 100 µg streptomycin, 100 units penicillin, 2 mM glutamine, 3 × 10⁻⁸ M selenium dioxide, 0.015 M HEPES, pH 7.2, 5 µg ml⁻¹ insulin, 5 µg ml⁻¹ transferrin) were also able to secrete the factor. Colony growth values are expressed as a % of control cultures (medium only). Values are means ± s.e.m. of triplicate determinations of seven experiments with different bone marrow and peripheral blood leukocyte samples. The number of bone marrow colonies in control cultures was 189-384 at day 7, and 73-134 at day 14 when plated at 10⁵ cells per dish. Colonies from peripheral blood leukocytes in control cultures were 14-152 when plated at 10⁶ cells per dish. Morphological examination of the colonies was on whole agar layers, fixed, dried and Giemsa-stained¹⁹.

(early) population than day-7 CFU-GM¹⁹. Thus, we conclude that the effect of CIL is directed to the more immature CFU-GM. To confirm this we added CIL to peripheral blood leukocyte CFU-GM at different days of culture. As expected, MT1 supernatant at 0.01% concentration completely inhibited CFU-GM growth only when added in the first 4 days of culture. The sensitivity of more immature bone marrow erythroid progenitors (BFU-E) and of pluripotent progenitors (CFU-GEMM) to the effects of CIL was comparable to that observed with day-14 CFU-GM, whereas less immature CFU-E were less sensitive (Table 2).

To rule out the possibility that the inhibitory effect observed on the haematopoietic progenitor cell proliferation was due to nonspecific cytotoxicity, we cultured a variety of human haematopoietic cell lines for at least 2 weeks in the presence of MT1 supernatant at 10% final concentration. T-cell lines (Molt 4 and Jurkat) as well as U-937 histiocytic lymphoma cells and K562 leukaemic cells were completely unaffected by the presence of the inhibiting factor. The growth of HL60 and ML3 human promyelocytic leukaemic cells was partially inhibited by the presence of the MT1 supernatant at the beginning of the test period, but the cells recovered and grew well after that. By contrast, all of the Epstein-Barr virus transformed lymphoblastoid cell lines and the B lymphoma cell lines, Daudi, Raji²⁰, ceased dividing after a few days of culture in the presence of CIL. After a period of time that varied between 6 and 10 days, depending on the cell line, all of the CIL-sensitive cells in the cultures treated with the factor had died, whereas the controls, grown in the presence of 10% 6TM medium continued normal growth. A positive correlation was found in human cell lines between expression of HLA-DR antigen and sensitivity to

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Table 2 Inhibitory effect of clone MT1 supernatant on the growth of bone marrow erythroid and pluripotent progenitor cells

Culture in presence of Medium (control) MT1 supernatant	Growth (% of control cultures) of		
	CFU-E 100	BFU-E 100	CFU-GEMM 100
10%	0	0	0
1%	0	0	0
0.1%	35 ± 22	0	0
0.01%	99 ± 12	2 ± 2	0
0.001%	—	74 ± 7	55 ± 9
0.0001%	—	98 ± 5	102 ± 12
6TM supernatant 10%	100 ± 11	102 ± 2	99 ± 12

Values are mean ± s.e.m. of three experiments with different bone marrow samples. The number of CFU-E-derived colonies in control culture ranged from 94–189 per 10^5 cells plated. Erythroid 'bursts' (BFU-E) ranged from 27–66 and 'mixed' colonies (CFU-GEMM), 3–7 per 10^5 cells plated. 1×10^5 low-density, non-adherent bone marrow cells were cultured in Iscove's modified Dulbecco's medium (Gibco) containing 0.8% methylcellulose (Dow), 30% fetal calf serum, 7% lymphocyte-conditioned medium as a source of burst-promoting activity (BPA), 10^{-4} M β -mercaptoethanol, 1.5 U ml $^{-1}$ sheep erythropoietin step 3 (Connaught)³¹ and various concentrations of MT1 supernatant. Fractions of MT1 supernatant filtered through a Sephadex G100 column were tested on CFU-E, BFU-E and CFU-GEMM. Only the fractions corresponding to a molecular weight of 85,000 contained inhibitory activity. Positive fractions were pooled and the factor used in one of the three experiments above at the appropriate reconstituted dilutions. CFU-E-derived colonies were scored after incubation for 7 days at 37 °C in a 5% CO $_2$ atmosphere. BFU-E- and CFU-GEMM-derived colonies were scored after 14 days of incubation. Lymphocyte-conditioned medium as a source of BPA was obtained by culturing peripheral blood leukocytes of prescreened donors at 1×10^6 per ml in Iscove's modified Dulbecco's medium containing 10% fetal calf serum and 1% phytohaemagglutinin for 7 days³².

Table 3 Characterization of CIL

Culture conditions	Growth of bone marrow CFU-GM day 14 (% of control cultures)
Medium (control)	100
MT1 supernatant 1% + GCT -CM 10%	1 ± 2
37 °C, 60 min	
MT1 supernatant 1% + trypsin, 0.5 mg ml $^{-1}$	92 ± 7
37 °C, 60 min	
MT1 supernatant 1%	123 ± 14
56 °C, 30 min	
GCT-CM 10%	119 ± 8
56 °C, 30 min	
MT1 supernatant 1% + GCT-CM 10%	
37 °C, 60 min	
56 °C, 30 min	101 ± 3
MT1 supernatant 1%	
100 °C, 15 min	91 ± 5
MT1 supernatant 1%	
(NH $_4$) $_2$ SO $_4$ 75%	1 ± 1
6TM supernatant 1%	
(NH $_4$) $_2$ SO $_4$ 75%	115 ± 6

Values are mean ± s.e.m. of three experiments with different bone marrow samples. The number of bone marrow day 14 colonies in control cultures of the three experiments was 26, 134, and 220, respectively, when the cells were plated at 10^5 cells per dish. The supernatant were from MT1 and 6TM cells cultured in synthetic medium. The experimental conditions are given in the legend to Table 1.

growth inhibition by CIL. The same correlation exists for bone marrow progenitor cells. The inhibitory effect of prostaglandin E on CFU-GM²¹ and of isoferritin on CFU-GM²², BFU-E and CFU-GEMM²³ has been shown to depend on the presence of some determinants of the cell-surface HLA-DR antigens. We are currently investigating this association.

The protein nature of CIL was demonstrated, based on its inactivation by heat (56 and 100 °C); its trypsin sensitivity and its precipitation by 75% (NH $_4$) $_2$ SO $_4$ (Table 3). Co-exposure of GCT-CM and CIL to 37 °C for 60 min followed by 30 min at 56 °C confirmed that the activity of CIL is not directed against GCT-CM and that only CIL is temperature-sensitive (Table 3). Gel filtration of the MT1 supernatant and subsequent testing of the eluted fractions for inhibitory activity on bone marrow day-7 CFU-GM identified the activity in the 85,000 molecular weight (MW) peak (Fig. 1). The same fractions were able to inhibit CFU-E, BFU-E and CFU-GEMM (Table 2). In the same conditions, fractions obtained after gel filtration of 6TM supernatant gave no inhibition.

The cell-surface phenotype of the parental clone 6TM and of the MT1 cells, as determined by monoclonal antibodies to human T antigens²⁴, was similar and no chromosomal markers or specific HLA antigens could be used to identify the MT1 clone definitively as a hybridoma. On the other hand, clone 6TM secretes no CIL into the culture medium.

The inhibitory pattern on myeloid progenitors, as well as the biochemical characteristics of CIL, are unlike those of other previously described factors that inhibit growth *in vitro* of bone marrow progenitor cells. Lactoferrin (MW 85,000–100,000), for example, indirectly affects the growth of CFU-GM by inhibiting the production of colony-stimulating factor (CSF) by monocytes²⁵, thereby precluding experimental demonstration of its effects in cultures stimulated maximally by exogenous CSF such as GCT-CM. On the other hand, prostaglandins E $_1$ and E $_2$ (ref. 26) (MW 1,000–1,500), and acidic isoferritin²⁷ (MW 450,000–500,000) inhibit colony formation by direct action on CFU-GM but inhibition even at high concentrations of the two factors reaches only 50% of control values, whereas CIL blocks CFU-GM completely. The inability of the MT1 supernatant to induce differentiation markers (NBT reduction, morphological changes) in HL60 cells, even if used at a 50% final concentration,

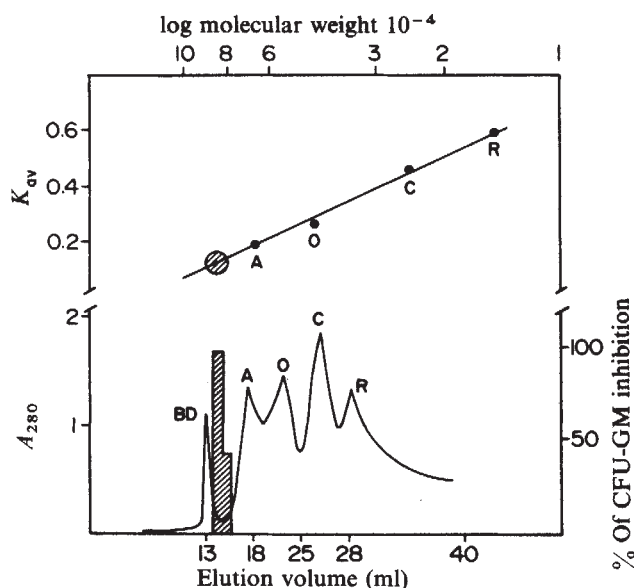


Fig. 1 Molecular weight determination of the lymphokine secreted by human T-cell clone MT1. The bottom graph shows the elution pattern of molecular weight markers. BD, Blue dextran; A, albumin (69,000); O, ovalbumin (43,000); C, chymotrypsinogen (25,000); R, ribonuclease A (13,700) filtered through a 0.9×55 cm Sephadex G-100 column at a flow rate of 2 ml hr $^{-1}$. The shaded region in the graph indicates the position of the fractions with inhibitory activity. The top graph is plotted based on the molecular weight markers and on the position of each elution peak. Supernatant of MT1 cells cultured in synthetic medium (see legend to Table 1) was passed through the column in the presence of 50 mg l $^{-1}$ of polyethylene glycol 6000 in PBS. The inhibitory activity, when tested on bone marrow day-7 CFU-GM, was contained in the eluted fractions corresponding to a molecular weight of 85,000.

distinguishes it from the differentiation-inducing factor (MW 35,000–55,000), a product³ of some stimulated T lymphocytes and T-cell lines. Finally, CIL could be distinguished from interferon (MW 15–60 000), which at high concentrations has an inhibitory effect on CFU-GM colony formation²⁸, as even undiluted CIL failed to protect human fetal skin fibroblasts or bovine brain fibroblasts in an assay²⁹ to measure the cytopathic effect of vesicular stomatitis virus (not shown).

A T-lymphocyte subpopulation from some severe aplastic anaemia patients¹⁴ and the pokeweed mitogen-stimulated T cells from healthy donors³⁰ produce a soluble factor that inhibits CFU-GM growth. Further, only patients who respond to immunosuppressive therapy with complete autologous haematological reconstitution have detectable CFU-GM suppressor T cells that release this inhibitor¹⁴. In the absence of a biochemical characterization of the factor produced by T cells of these patients, no direct comparison with CIL can be made at this stage.

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Cross-bridge interaction with oppositely polarized actin filaments in double-overlap zones of insect flight muscle

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An unresolved problem in understanding muscular contraction is why the internal resistance to sarcomere shortening increases progressively during contraction¹. We have addressed this problem here by investigating the movement of detached acting filaments in the sarcomeres of insect flight muscle. The final position of the detached actin filaments shows that they were able to slide freely into regions where they have the wrong polarity to interact actively with myosin (double-overlap zones) but where they prevent the exertion of force by cross-bridges between myosin and the correctly polarized acting filaments. These observations indicate that the isometric tension at all sarcomere lengths is directly proportional to the number of cross-bridges in the region of single-overlap of correctly polarized actin and myosin filaments. The decrease in tension as sarcomeres shorten is thus the result of the decrease in the number of effective cross-bridges as actin filaments slide into regions where they are of the wrong polarity to form cross-bridges, and where they inhibit the existing cross-bridges.

When insect fibrillar flight muscles are stretched in rigor, the actin filaments break away from the Z lines in some sarcomeres, although the continuity of the myofibrils is maintained because the C filaments anchor the myosin filaments to the Z lines^{2–4}. The large mechanical strength of the C filaments^{4,5} makes it possible to study the contractile mechanism of insect fibrillar muscle in completely unloaded conditions. The detached actin filaments in stretched sarcomeres can slide freely after activation of the muscle, and it was expected that the final positions of the broken actin filaments would provide new information about the mechanism of filament movement.

Flight muscles of the honey bee, *Apis mellifera*, were slightly

stretched (up to 10% of the rest length)⁶ and then glycerinated⁷ in 50% glycerol, 50% water, 20 mM Tris pH 7.0. After glycerination the muscles were transferred into a rigor solution⁸ (5 mM MgCl₂, 5 mM EGTA, 100 mM KCl and 20 mM Tris pH 7.0) and moderately stretched (up to about 20% of rest length) again. The stretched muscles were then treated with an activating solution⁸ of 5 mM ATP, 5 mM MgCl₂, 5 mM Ca EGTA, 100 mM KCl and 20 mM Tris pH 7.0, and fixed by the addition of 1/10 volume Tris-buffered 25% glutaraldehyde. The specimens were post-fixed in osmium tetroxide and processed for electron microscopy. In our experimental system the disturbing effects of muscular contraction—the crumpling or folding of the myosin filaments along the Z lines and the large increase of lateral spacing between filaments—were eliminated.

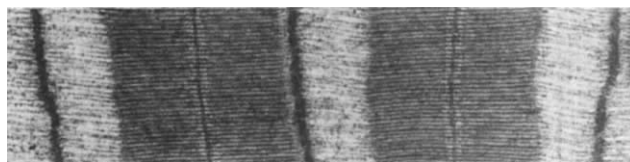


Fig. 1 Insect flight muscle stretched in rigor. Asymmetrically and symmetrically elongated sarcomeres. The broken actin filaments remained in their original position without additional activation. $\times 9,500$.

In the experimental conditions described above, the actin filaments broke at either one or both ends of the A bands, producing asymmetrically or symmetrically elongated sarcomeres. The broken actin filaments remained in their original position as long as the stretched muscles were not activated (Fig. 1). On activation the broken actin filaments moved across the M line in one of two different ways. In the asymmetrically elongated sarcomeres, the broken actin filaments moved almost completely to the intact half-sarcomeres (see Fig. 2 inset). A very narrow overlap zone remained only along the M line (Fig. 2). Thus the damaged half-sarcomere became an H zone, and the intact half-sarcomere became a double-overlap zone. Presumably only the very last cross-bridges at the border of the M region could provide the force to move the broken actin filaments

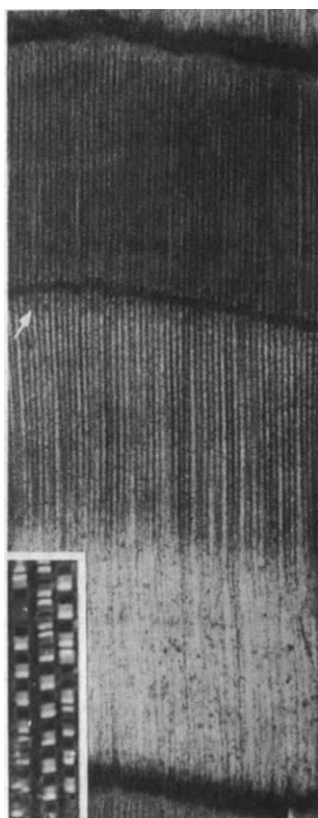


Fig. 2 Asymmetrically elongated sarcomere after activation. The broken actin filaments were almost completely transported into the intact half-sarcomere. A very narrow overlap zone (arrow) remained only along the M line. $\times 26,000$. Inset: the same area as seen in an optical microscope. $\times 1,000$.

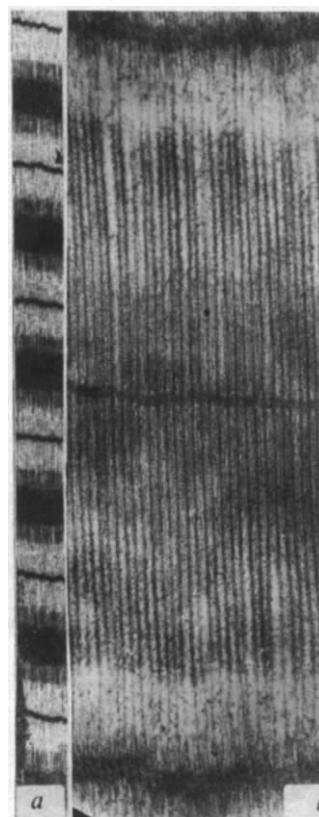


Fig. 3 Symmetrically broken sarcomeres after activation. *a*, Dense contraction bands developed in the middle of the A bands. $\times 4,000$. *b*, The contraction band consists of completely overlapped broken actin filaments. $\times 29,000$.

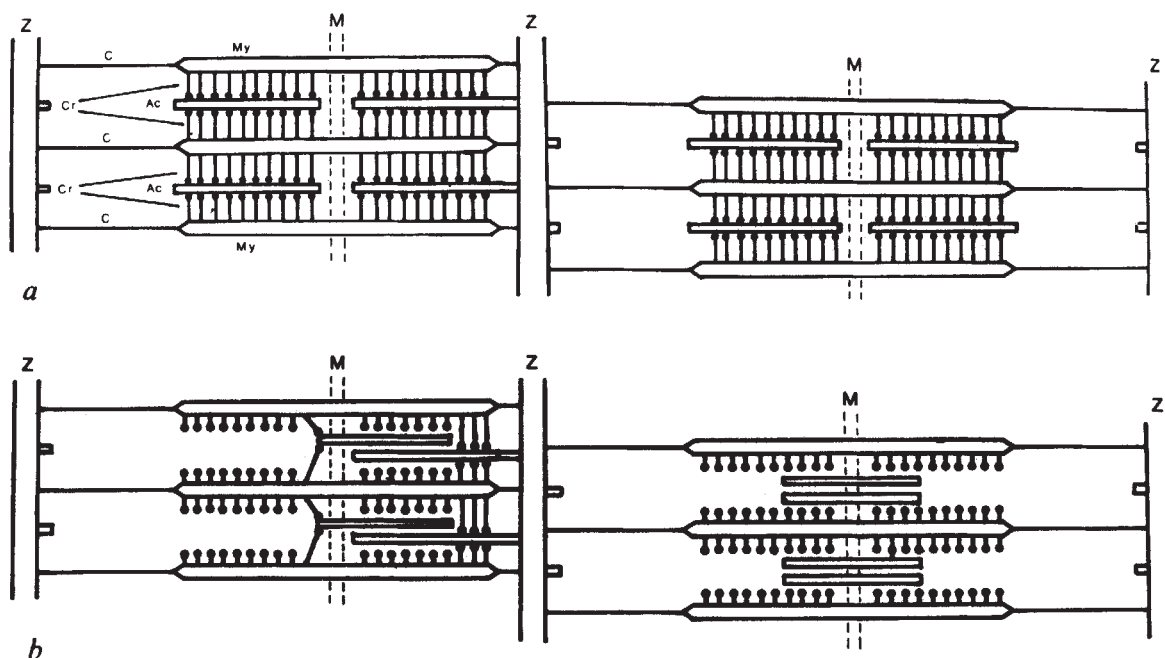


Fig. 4 Schematic diagrams of critical stages of broken actin filament positions and cross-bridge interactions with actin filaments before and after activation in both asymmetrical and symmetrical sarcomeres. *a*, Illustrates the situation shown in Fig. 1. The actin (Ac) filaments break along the Z lines as a result of stretching in rigor, but the gaps between the Z lines and A bands are bridged over by C filaments which connect the myosin (My) filaments to the Z lines. All of the cross-bridges (Cr) are attached to the actin filaments and this rigid connection prevents the motion of broken actin filaments. *b*, The first sarcomere illustrates the situation shown in Fig. 2. In asymmetrically elongated sarcomeres only a few cross-bridges are necessary to propel the broken actin filaments into the almost complete double-overlap zone during activation. The cross-bridge interaction with actin filaments is limited to the normal overlap zones. The second sarcomere illustrates the situation shown in Fig. 3. In symmetrically elongated sarcomere the molecular interaction of completely overlapped actin filaments inhibits the generation of force by the cross-bridges.

into the double-overlap zone, which suggests that the viscous resistance to the relative sliding motion of actin and myosin filaments in each half-sarcomere is negligibly small in comparison with the forces generated by a few cross-bridges⁹. Furthermore, neither the opposite polarities of the actin filaments and the cross-bridges nor the increased filament concentration in the intact half-sarcomeres retarded the motion of broken actin filaments. In symmetrically elongated sarcomeres, contraction bands developed in the middle of the A bands (Fig. 3a) consisting of completely overlapping actin filaments (Fig. 3b). The broken actin filaments apparently stopped moving when the complete double-overlap zone developed in the middle of the A band.

Thus, only a few cross-bridges in the normal overlap zone are required to push broken actin filaments fully into the form of a double-overlap zone in the opposite half-sarcomere. However, although in the double-overlap zone of symmetrically broken sarcomeres many more cross-bridges face correctly polarized actin filaments, the filaments stop moving in mid-sarcomere as soon as normal single-overlap is no longer possible. Apparently only those bridges that are located in the normal overlap zones can generate effective force. Active tension must

therefore fall to zero in a contracting muscle when the complete double-overlap zones develop. That this conclusion is also valid for vertebrate skeletal muscle is supported by the observation^{1,10} that the active tension of striated muscle decreases to zero at lengths where complete double-overlap zones are expected to develop.

It is puzzling that although the incorrectly polarized actin filaments apparently interact so intimately with cross-bridges as to inhibit their ability to exert force on correctly polarized acting filaments, they can still slide past 10–40 cross-bridges when propelled by only one or two active cross-bridges. Our findings are summarized schematically in Fig. 4.

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Loss of alleles at loci on human chromosome 11 during genesis of Wilms' tumour

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Evidence that recessive cellular alleles at specific chromosomal loci are involved in tumorigenesis has been recently shown by work on tissues from patients with retinoblastoma, a neoplasm of embryonic retina whose predisposition is inherited as an autosomal dominant trait¹. A comparison of germ-line and tumour genotypes at loci on human chromosome 13, defined by restriction fragment length polymorphisms, showed that loss of the chromosome bearing the wild-type allele at the *Rb-1* locus occurred frequently in the development of retinoblastoma. We report here results of similar studies of another embryonal neoplasm, Wilms' tumour of the kidney. Examination of germ-line and tumour genotypes from seven patients showed that five cases were consistent with the presence on human chromosome 11 of a locus in which recessive mutational events are expressed after abnormal chromosomal segregation events during mitosis.

Wilms' tumour, the most common abdominal neoplasm in children, occurs in both hereditary and nonhereditary forms², and so may be a member of a group of paediatric tumours, including retinoblastoma, neuroblastoma and medulloblastoma, which have a significant genetic component in their aetiology. The autosomal dominant heritable form is often associated with other specific congenital malformations such as aniridia, genitourinary abnormalities and mental retardation³. Published reports^{1–7} on Wilms' tumour patients imply that mutant forms of one or more loci contained within the 11p13 band on chromosome 11 predispose to the formation of this tumour. The mutations are not, however, sufficient to elicit the neoplasia since discrete foci are seen amidst the background of normal kidney, even in cases in which the 11p13 band of one homologue is

missing in the germ line. Thus, a second, post-zygotic event must also be involved. In retinoblastoma this is loss of all or part of the homologous wild-type chromosome 13 during mitosis of the retina¹ allowing expression of initial predisposing recessive germ-line mutations. We aimed to find out whether similar mechanisms have a role in the development of Wilms' tumour.

We isolated high molecular weight DNA from seven primary Wilms' tumours and corresponding normal tissue (either from peripheral blood leukocytes or from unaffected kidney where possible), digested these samples with appropriate restriction endonucleases, separated the resultant fragments by electrophoresis through agarose gels and transferred the DNA fragments to nylon membranes¹. Each of these paired samples was then independently hybridized to four recombinant DNA probes which have been mapped to subregion p15 of the short arm of chromosome 11 (ref. 8), and which reveal restriction fragment length polymorphism. The probe pADJ-762 was a random human genomic sequence which reveals polymorphic alleles, in *BclI*-digested DNA, of 11.6 and 4.2 kilobases (kb) as well as a constant band of 6.9 kb (refs 8, 9). It also showed an independent polymorphism, in *MspI*-digested DNA, with alleles of 2.3 and 1.8 kb (refs 8, 9). The probe pHins-310 is homologous to the 5'-flanking region of the human insulin gene¹⁰, and shows polymorphic alleles of various lengths due to the insertion or deletion of blocks of DNA¹¹. The probe JW151 has homology to the human β -globin locus¹² and reveals two independent restriction fragment length polymorphisms, one with alleles of 8.0 and 7.2 kb representing the γ G portion of the locus, and one with alleles of 3.5 and 2.7 kb representing the γ A portion of the locus. The probe pTBB2 is homologous to the transforming gene of the Harvey sarcoma virus¹³ and, in *TaqI*-digested DNA, reveals polymorphic alleles of various lengths due to insertion or deletion of blocks of DNA^{8,13}. Each of these probes has been independently mapped to the chromosomal region 11p15→11pter (refs 8, 14, 15).

Figure 1 displays representative results of the constitutional and tumour genotypes of three of the patients. Patient 1 was constitutionally heterozygous (that is, revealed both co-dominant alleles) at four loci on chromosome 11p, those defined by pADJ-762 in *BclI*-digested DNA, pHins-310 in *PvuII*-digested DNA, and the γ G and γ A portions of the β -globin locus defined by JW151 in *HindIII*-digested DNA. One of the alleles at each of these loci was not apparent in primary tumour tissue from this patient. Although tumour cell karyotypes were not obtained in this case, it is unlikely that these allele losses resulted from a deletion of the terminal portion of the short arm of chromosome 11 since the hybridization intensity of each

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Table 1 Alleles at chromosome 11 loci in constitutional (C) and tumour (T) tissue from seven cases of Wilms' tumour

Alleles present							
Patient	Tissue	pADJ-762		pHins-310 (<i>PvuII</i>)	JW151 (<i>HindIII</i>)		pTBB2 (<i>TaqI</i>)
		(<i>MspI</i>)	(<i>BclI</i>)		^γ G	^γ A	
1	C	2,2	1,2	1,2	1,2	1,2	2,2
	T	2,2	2,2	2,2	1,1	1,1	2,2
2	C	2,2	2,2	1,2	1,2	1,2	2,2
	T	2,2	2,2	2,2	2,2	1,1	2,2
3	C	2,2	2,2	1,3	2,2	1,1	—
	T	2,2	2,2	1,3	2,2	1,1	—
4	C	1,2	1,1	1,3	1,2	1,1	2,2
	T	2,2	1,1	3,3	1,1	1,1	2,2
5	C	2,2	1,2	2,3	1,2	1,1	2,2
	T	2,2	1,1	3,3	2,2	1,1	2,2
6	C	1,2	2,2	1,2	1,2	1,1	1,2
	T	2,2	2,2	2,2	1,1	1,1	2,2
7	C	2,2	1,2	1,2	2,2	2,2	—
	T	2,2	1,2	1,2	2,2	2,2	—

Alleles for each locus are named with 1 as the longer allele and 2 or 3 as the shorter. Constitutional tissue from patients 1, 2, 3, 4, 5 and 7 was peripheral blood lymphocytes; in patient 6 normal kidney was obtained. The patients and their reference numbers are: patient 1, CHMC 433256; patient 2, CHMC 385346; patient 3, CHMC 443779; patient 4, CHMC 363775; patient 5, CHMC 385557; patient 6, CHMC 286794; patient 7, CHMC 407291. —, Not examined.

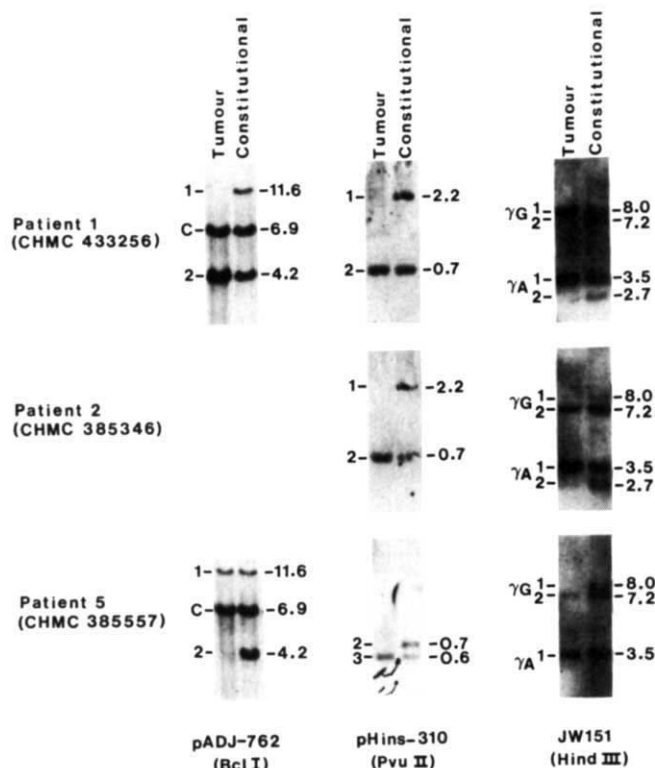


Fig. 1 Loss of heterozygosity at loci on human chromosome 11p in three cases of Wilms' tumour. DNA from paired tissues was digested to completion with the indicated restriction endonuclease, subjected to agarose gel electrophoresis, transferred to Zeta-por membranes (AMF-Cuno) and hybridized to the 32 P-labelled nick-translated probes pADJ762 (refs 8, 9), pHins-310 (refs 8, 10, 11), or JW151 (refs 8, 12). All methods as in ref. 1. Each tumour was obtained prior to any therapy. Alleles at each locus are designated 1, 2 or 3 according to decreasing length; C indicates a common, invariant band. Numbers to the right of each autoradiograph indicate the molecular size of the indicated allele in kilobases, derived from standards run with each gel.

of the alleles remaining in the tumour tissue was greater than that obtained from corresponding alleles in normal tissue from this patient. These results suggest that this loss of alleles at multiple loci during Wilms' tumour oncogenesis was not due to a simple deletion event and may, by analogy with the previous analysis of retinoblastoma tumours¹, indicate that loss of one chromosome 11 homologue has been effected with subsequent reduplication of the chromosome carrying the initial predisposing recessive mutation.

Similarly, patient 2 was constitutionally heterozygous at the locus homologous to pHins-310 and at the γG and γA portions of the β-globin locus defined by JW151. Each of these loci had become homozygous in primary tumour tissue from this patient and the hybridization intensities of the alleles remaining in the tumour were again greater than those of the corresponding constitutional alleles. Karyotypic analysis of tumour cells from this patient showed no discernible deletion of an 11p homologue (data not shown). Patient 5 was also constitutionally heterozygous at the loci homologous to pADJ-762 and pHins-310 as well as to the γG portion of the β-globin locus homologous to JW151. Each of these loci became homozygous in tumour tissue from this patient. Although technical considerations precluded analysis of the differential hybridization intensities in this case, karyotypic analysis of these tumour cells did not show any obvious deletion of either 11p homologue.

Thus, in these three patients, the allele losses described here are probably not attributable to a deletion of chromosome 11. A more likely explanation, based on these data and previous work with retinoblastoma¹, is that a mitotic segregation event has occurred in each predisposed kidney cell such that one chromosome homologue was lost and the remaining homologue was reduplicated during the process of tumorigenesis; we infer that the remaining chromosomes are each defective at the Wilms' tumour locus contained within band 11p13. It is also possible that mitotic recombination events occurred between the most proximal marker locus (defined by JW151) and the centromere such that one recombinant chromosome remained in these tumours. We hypothesize that such events would have break points between the Wilms' locus and the centromere resulting in homozygosity for recessive mutant alleles at the Wilms' locus, similar to the retinoblastoma case Rb-412 (ref. 1). Distinguishing between these mechanisms will require the development of probes homologous to loci between band p13 and the centromere.

Table 1 presents all of the genotypic data obtained in this study. Patients 1, 2, 4, 5 and 6 were constitutionally heterozygous at 4, 3, 3, 3 and 4 loci, respectively. In each case, at each locus, one of the two co-dominant alleles was lost in the process of tumorigenesis, thus predisposed kidney cells in patients 4 and 6 may also have undergone the same sorts of mitotic events more fully described for patients 1, 2 and 5 in Fig. 1. Two points are worth noting in this regard: first, tumour karyotypes from patient 6 showed no obvious 11p deletion, and second, the normal tissue analysed from patient 6 was non-neoplastic kidney adjacent to the tumour. Patients 3 and 7 were constitutionally heterozygous at 1 or 2 loci, respectively; in each case heterozygosity at these loci was retained in the corresponding tumour tissue. These latter results suggest that these rather gross chromosomal mechanisms may not occur in all tumours. It is, of course, possible that these two tumours had become homozygous over a small region of the chromosome including the Wilms' locus and that these events were too localized to be detected with probes which do not map very near to the disease locus. Probes which represent loci physically close to the Wilms' locus will have to be developed to study this in more detail. Nonetheless, five of the seven independent primary tumours examined in this study appear to have undergone mitotic events which could serve to unmask initial predisposing recessive mutations. To test whether the loss of heterozygosity in these Wilms' tumours was specific to chromosome 11 or part of a more general reduction to homozygosity, we hybridized the same filters from which these data were drawn to two probes homologous to

human chromosome 13, p9A7 and p7F12 (ref. 1). Although alleles at these loci are lost during the oncogenesis of retinoblastoma¹, in each Wilms' patient who was constitutionally heterozygous (five of the seven examined here), the corresponding tumour tissue remained heterozygous (data not shown).

Thus, the work reported here shows that abnormal mitotic events that allow expression of recessive mutations predisposing to cancer¹ are involved in more than one tumour type and affect more than one chromosome and that the strategies previously proposed¹ for mapping recessive genes involved in the oncogenesis of various tumour types can be valuable in identifying the parental chromosome carrying the predisposing defect and in genetic counselling. The possibility of mapping such genes in many different tumours could lead to their eventual isolation and molecular analysis.

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Development of homozygosity for chromosome 11p markers in Wilms' tumour

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Somatic alterations in the genome are found in many human tumours. Chromosome rearrangements¹ or base substitutions² that activate cellular oncogenes appear to act dominantly. In contrast, recessive alleles apparently contribute to childhood retinoblastoma^{3–5}, as homozygosity (or hemizygosity) for chromosome 13 is often established in tumours, by either mitotic nondisjunction or recombination⁵. Parallels exist between retinoblastoma and childhood Wilms' tumour (WT). Retinoblastoma is often inherited and accompanied by a deletion of chromosome 13 (band q14), while WT is occasionally associated with aniridia and deletion of chromosome 11 band p13 (refs 6, 7). Most Wilms' tumours are sporadic and not accompanied by these findings, although interstitial deletion of chromosome 11 in tumour, but not normal, cells has been reported⁶. In view of these parallels, we compared constitutional and tumour

DNA from WT patients by using chromosome 11p DNA probes. We report here that although heterozygosity in constitutional DNAs was often preserved in tumour DNAs, one case developed homozygosity for chromosome 11p markers in tumour cells, implying the involvement of chromosomal events in revealing a recessive WT locus. This observation suggests the action of such general mechanisms in a tumour other than retinoblastoma.

All WT patients studied were of the typical sporadic variety and were free of aniridia (see Table 1 for ages at diagnosis). All tumours were unilateral in origin. High-molecular weight DNAs were prepared from peripheral blood white cells⁹ and from tumour samples¹⁰ removed at surgery. Transfection of these tumour DNAs into NIH 3T3 cells¹¹ did not demonstrate the presence of assayable dominantly acting transforming genes. To explore the potential role of other mechanisms in tumour formation, we analysed DNA polymorphisms in constitutional and tumour samples by blot hybridization¹² with a variety of cloned DNA probes derived from the short arm of chromosome 11. Constitutional DNAs were first studied for informative markers, that is, those for which heterozygosity could be established. On identification of a suitable marker, tumour DNA was analysed. The informative markers included several in the β -globin gene complex and the c-Harvey-ras1 oncogene² (c-Ha-ras1). Whereas the β -globin complex was initially assigned to 11p11–12.08 (ref. 13), recent evidence has indicated a more distal location¹⁴, probably 11p15 (ref. 15). c-Ha-ras1 has been localized to 11p, most likely 11p15.1–15.5 (refs 16–19). Probes for the 5'-flanking region of insulin (11p15)²⁰ and for parathyroid hormone²¹ (11p15; B. Zabel, personal communication) were also used, but these provided no additional informative data. The results are summarized in Table 1.

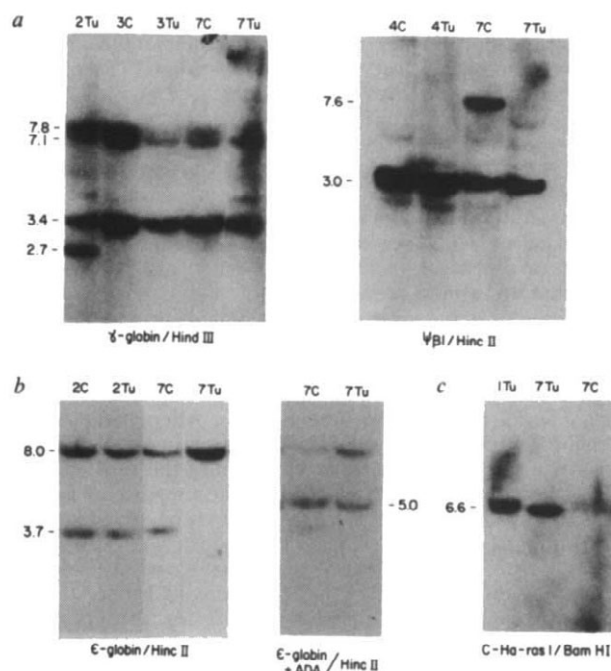
In all seven cases at least one restriction fragment length polymorphism was detected in constitutional DNAs either with the various probes from the β -globin complex or with c-Ha-ras1. In six cases heterozygosity detected by a probe was preserved in the tumour DNA; in three of these cases, heterozygosity at two or more sites was observed to be identical in constitutional and tumour samples. In one case (7 in Table 1), however, heterozygosity in the constitutional DNA detected by three globin probes and by the c-Ha-ras1 probe was contrasted with homozygosity at these sites in tumour DNA. Neighbouring normal kidney tissue DNA was heterozygous at these sites (see Table 1). Representative blot hybridization results for this and other cases are presented in Fig. 1. Cytogenetic analysis of circulating lymphocytes of case 7 revealed that they were normal. No cultured tumour cells were available for study. The mother of case 7 was heterozygous for the β -globin haplotype, +----- [5'- ϵ - γ - ψ 1-3']. Homozygosity for the paternal chromosome 11, including the c-Ha-ras1 locus, was established in the tumour. Case 7 constitutional and tumour DNAs were homozygous at insulin and parathyroid hormone loci (not shown).

Loss of heterozygosity for the 11p markers in case 7 could reflect chromosome loss via nondisjunction in the tumour, chromosome loss followed by reduplication of the other homologue, or somatic recombination, all of which have been deduced in analogous studies of retinoblastoma⁵. Chromosome loss alone (or of 11p) appears not to be the underlying mechanism on the basis of gene dosage in the blot hybridizations (see Fig. 1a–c, particularly *HincII* digests). Hybridization to a single-copy sequence located on another autosome was used to demonstrate that equivalent amounts of DNA were present in the critical gel lanes (Fig. 1b). The experimental findings imply chromosomal loss followed by reduplication rather than loss alone. Due to the absence of informative markers in the proximal portion of 11p, somatic recombination cannot be excluded as an alternative mechanism for development of homozygosity.

It is formally difficult to exclude similar phenomena occurring in this tumour on chromosomes other than 11 or the segment 11p. To obtain evidence against this unlikely possibility, DNA markers on other chromosomes were evaluated. Heterozygosity

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Fig. 1 Autoradiograms of representative blot hybridizations. DNA samples were analysed as described in Table 1. The origin of each DNA is given at the top of each lane [case no.; constitutional (C) or tumour (Tu)]. Fragment lengths are given in kilobases (kb). *a*, γ -Globin and $\psi\beta 1$ region polymorphisms. Note the preservation of heterozygosity at γ loci in tumour samples of cases 2 and 3 and development of homozygosity at γ -globin and $\psi\beta 1$ loci in case 7. *b*, Polymorphism at the ϵ -globin locus. Note the preservation of heterozygosity in the tumour sample of case 2 and contrast between constitutional and tumour samples of case 7. After hybridization and autoradiography of the filter containing 7C and 7Tu DNAs with the ϵ -globin probe (middle two lanes), adenosine deaminase (ADA) cDNA³³ (localized to chromosome 20) was applied for a second hybridization (right-hand lanes). The comparable intensity of the 5.0-kb adenosine deaminase fragment provides an internal check that equivalent amounts of DNA were present in the gel lanes containing the *HincII* digests. Residual hybridization of the ϵ -globin-specific fragments is still visible. *c*, Polymorphism at the c-Ha-ras1 oncogene. Heterozygosity of case 1 tumour and case 7 constitutional DNAs is depicted and contrasted with homozygosity in case 7 tumour DNA.



in the tumour was detected by probe pHU10 (*XmnI* digest, chromosome 13)²², DOSLC 2 (*MspI* digest, chromosome 20)⁵ and DOSLC 3 (*MspI* digest, chromosome 15)⁵. No polymorphism in constitutional or tumour DNA was detected with probe pAW101 (*EcoRI* digest, chromosome 14)²³, pHU26 (*BglII* digest, chromosome 13)²², pHUB8 (*EcoRI* and *HindIII* digests, chromosome 13)²², or antithrombin III cDNA (*PstI* digest, chromosome 1)²⁴. The maintenance of heterozygosity in the tumour for DNA markers located on chromosomes other than 11 suggests that development of homozygosity for the 11p

markers is of particular significance in this situation. The maintenance of heterozygosity for the 11p markers in normal kidney tissue indicates that establishment of homozygosity did not occur during embryogenesis of the kidney but rather at a later date. We suggest that the development of homozygosity was related to development of the Wilms' tumour. It seems likely, although not proven, that establishment of homozygosity for the 11p markers used here parallels similar phenomena at the site of the presumed WT locus (11p13). According to this model, one chromosome 11 homologue of case 7 would carry a defective

Table 1 Restriction fragment length polymorphisms in Wilms' tumour patients

Case	Age	Source	β-Globin complex markers					c-ras
			ε (<i>Hinc</i> II)	γ (<i>Hind</i> III)	ψβ1(<i>Hinc</i> II)	β(<i>Ava</i> II)	3'β(<i>Bam</i> HI)	(<i>Bam</i> HI)
(1)	5	C		--/--	--/--	+/+	+/–	Heterozygous
		Tu			--/--		+/–	Heterozygous
(2)	3	C	+/–	++/--	--/--	+/–	+/+	
		Tu	+/–	++/--	--/--	+/–	+/+	
(3)	4	C	+/–	++/--	--/++	+/+	+/+	
		Tu	+/–	++/--	--/++	+/+	+/+	
(4)	61/2	C	–/–	+/+–	++/++		+/–	Homozygous
		Tu	–/–	+/+–	++/++		+/–	Homozygous
(5)	3	C	+/+	--/--	--/--	+/+	+/+	Heterozygous
		Tu	+/+	--/--	--/--	+/+	+/+	Heterozygous
(6)	31/2	C		--/--	--/–+	+/+	+/+	
		Tu			--/–+			
(7)	5	C	+/–	++/--	--/++	+/+	+/+	Heterozygous
		Tu	–/–	++/--	++/++	+/+	+/+	Homozygous
		N1-kidney	+/–	++/--	--/++			Heterozygous
	Mother	C	+/+	--/--	--/--			Heterozygous

Wilms' tumour cases with their ages at presentation are listed in arbitrary order. Constitutional (C, peripheral white blood cell) and tumour (Tu) DNAs were digested with an appropriate restriction enzyme, electrophoresed in 0.7–1.0% agarose, transferred to nitrocellulose filters¹², and hybridized with nick-translated probes of specific activities 1–3×10⁸ d.p.m. μg^{–1}. Prehybridization and hybridization conditions were as described elsewhere⁹. All filters were washed in stringent conditions (0.1×SSC at 68°C) before autoradiography with intensification for 24–96 h²⁸. Polymorphisms analysed were those within the ϵ -globin gene²⁹, the γ - and $\psi\beta 1$ -globin genes³⁰, $\psi\beta 1$ ²⁷, the β -globin gene²⁹, 3' to the β -globin gene³¹, and flanking the c-Ha-ras1 oncogene³². For single site polymorphisms, (+) indicates the presence of a site, (–) its absence. The nomenclature for the γ -globin and $\psi\beta 1$ polymorphisms is described in ref. 29. The (/) separate the two chromosome homologues. The length polymorphism detected in the c-Ha-ras1 locus is referred to merely as homozygous or heterozygous with the major *BamHI* allele = 6.6 kilobases (see Fig. 1c)³². Those loci at which individuals were heterozygous are enclosed in boxes. N1, normal kidney.

(or deleted), recessively acting WT locus. Subsequent loss of the normal homologue and duplication of that bearing the mutant locus would lead to tumour formation. The original mutation in the WT locus might be present in germ-line cells, or might arise somatically in the kidney, before the chromosome loss and duplication event. These data, together with the previous report that c-Ha-ras1 is localized outside the aniridia-Wilms' deletion^{14,19}, do not suggest involvement of this oncogene in WT formation. The presence of the putative WT locus and c-Ha-ras1 on the same chromosome may well be fortuitous.

Failure to witness development of homozygosity at 11p markers in six of the seven cases studied suggests more localized chromosomal phenomena, perhaps within 11p13 itself. The lack of available and informative markers in this and more proximal regions prevents identification of somatic recombination events neighbouring the putative tumour locus. Alternatively, additional mechanisms not involving reduction to homozygosity at this locus may contribute to development of this tumour. Furthermore, trivial explanations, such as the presence of normal cells within some tumour specimens, cannot be excluded.

We cannot accurately estimate the frequency of events such as that seen in case 7 among WT patients. However, from our results and those reported in the accompanying papers²⁵⁻²⁷, we conclude that the chromosomal events first described in retinoblastoma are not limited to that tumour, but are involved also in Wilms' tumour. These observations indicate the more widespread importance of these events in oncogenesis.

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Loss of a Harvey ras allele in sporadic Wilms' tumour

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Genomic changes within chromosome band 11p13 appear to have a role in the initiation of Wilms' tumour¹⁻⁷. The human Harvey ras oncogene, c-Ha-ras1, has been located by Jhanwar *et al.*⁸ immediately adjacent to this region at band 11p14.1, although several groups have assigned the gene more distally at band 11p15 (refs 9-11). We have examined tumour DNA from two cases of sporadic Wilms' tumour, and report here that in both cases one of the two constitutional c-Ha-ras1 alleles was absent. One tumour had a reciprocal translocation between the short arm of chromosome 11 (at band 11p13), and the long arm of chromosome 12, with no visible loss of chromosomal material. The loss of a c-Ha-ras1 allele in association with this translocation indicates that a submicroscopic deletion had occurred. The resulting hemizygosity may have had a role in tumour initiation. Our results indicate that the c-Ha-ras1 gene and the 'Wilms' tumour locus' may be in close proximity. It would, therefore, be premature to exclude the possibility that these two sites are functionally related.

Tissue was taken from two sporadic Wilms' tumours. Analysis of tumour 1 after 8 and 10 days in culture revealed two cell types: the predominant type having an apparently balanced reciprocal translocation 46,XX,t(11;12)(p13;q13) (Fig. 1), and a minor type with an apparently normal karyotype⁷. After 18 weeks in culture all cells contained an additional chromosome 8 and the normal line had disappeared. This chromosome addition was not related to the development of the original tumour *in vivo* as it occurred after prolonged growth *in vitro*. The lack of karyotypic evolution with respect to chromosome 11 after growth *in vitro* indicates that the c-Ha-ras1 genotype reflected faithfully the genotype of the original tumour. Eight-day cell cultures from tumour 2 contained at least two distinct karyotypic populations: most showed marked hypodiploidy with very contracted chromosomes, while a minor population had an apparently normal karyotype. We were unable to interpret further the chromosome pattern from the hypodiploid cells. Peripheral lymphocytes from both patients had normal karyotypes.

Figure 2a shows the pattern of hybridization of c-Ha-ras1 probe (pEJ6.6)¹² to BamHI digests of leukocyte DNA from five normal individuals. In four control samples, two BamHI restriction fragments of equal intensity were observed (lanes 4, 8, 10 and 12) while the fifth control (lane 6) contained a single intense band. Differences in the lengths of these BamHI fragments are believed to be due to the presence of a variably repeated 28-base pair (bp) consensus sequence localized outside the coding region of the c-Ha-ras1 gene¹³. Our results are consistent with previous work indicating that BamHI restriction fragment length polymorphisms correspond to allelic fragments containing c-Ha-ras1 (ref. 14).

Hybridization patterns of the c-Ha-ras1 probe to Southern blots of BamHI-digested DNA from tumour 1 (cells were cultured for 18 weeks before DNA isolation; see above) and leukocyte DNA from the same patient are shown in Fig. 2b (lanes 2 and 1, respectively). Of the two allelic BamHI fragments in the leukocyte DNA (7.6 kilobases (kb) and 8.6 kb), only one was represented in the tumour (8.6 kb). Furthermore, the tumour BamHI allele at 8.6 kb (Fig. 2a, lane 2) does not co-migrate with either of the mothers' alleles at 7.1 kb and 7.6 kb (Fig. 2a, lane 4). We conclude, therefore, that one c-Ha-ras1 allele has

Fig. 1 Partial karyotype of Wilms' tumour 1, demonstrating the reciprocal translocation between the short arm of chromosome 11 and the long arm of chromosome 12. The chromosome break points are indicated on the idiogram.

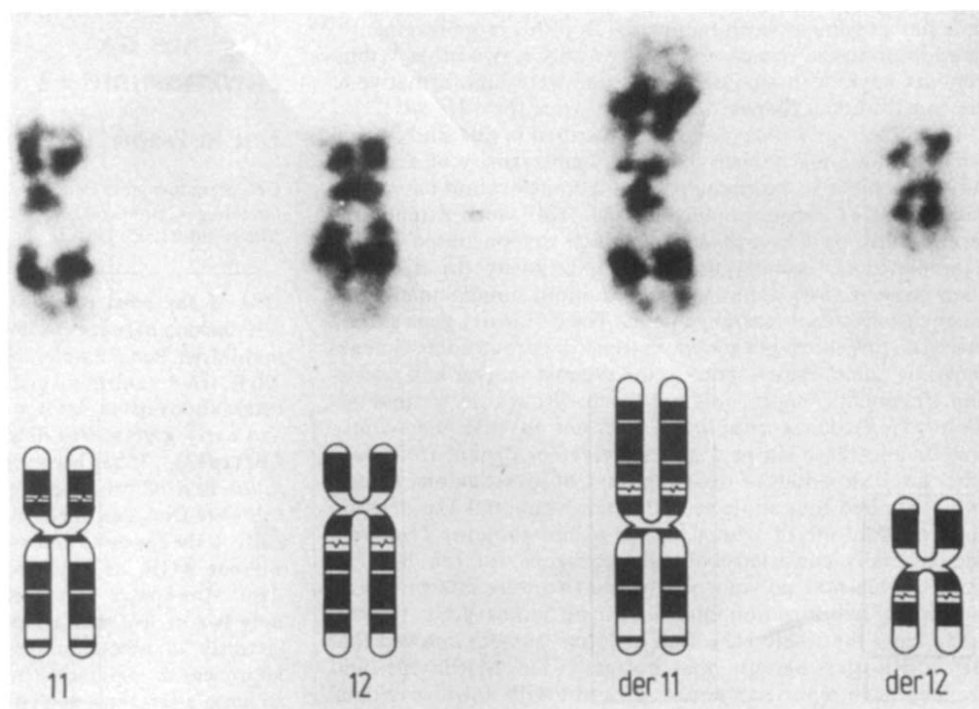
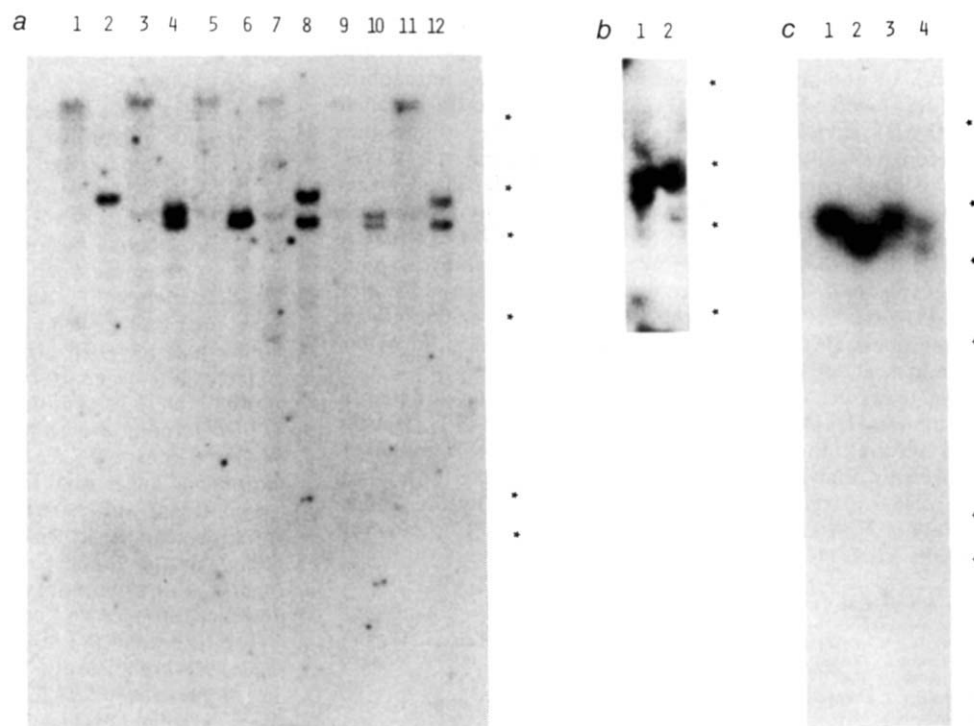


Fig. 2 Detection of sequences homologous to c-Ha-ras1 in human DNA samples. *a*, Even and odd numbers represent *Bam*HI and *Eco*RI digests respectively. Lanes 1, 2, tumour 1 (18-week cell culture); lanes 3, 4, mother of patient 1; lanes 5, 6; 7, 8; 9, 10; and 11, 12 are leukocyte DNA samples from normal individuals. *b*, *Bam*HI digests of: lane 1, leukocyte DNA from patient 1; lane 2, DNA from tumour 1 (18-week culture). *c*, *Bam*HI digests of: lanes 1, 2, leukocyte DNA from father and mother of patient 2, respectively; lane 3, tumour DNA from patient 2; lane 4, leukocyte DNA from patient 2. Less than 10 μ g of DNA was analysed in *c*, lane 4, thus the bands are less intense. λ -HindIII size markers are indicated by asterisks alongside each panel.



Methods: High-molecular weight cellular DNA²⁰ (~10 μ g) was digested for 12 h with either *Bam*HI (5U μ g⁻¹) or *Eco*RI (5U μ g⁻¹) and analysed by Southern blot hybridization.

Digests were assessed for completion in parallel digests containing pBR322 DNA. Southern blots were hybridized with ³²P-labelled nick-translated²¹ pEJ6.6 plasmid¹² and washed stringently in 0.1 $\times$ SSC, 0.5% SDS at 68 $^{\circ}$ C.

been lost in the Wilms' tumour, and that in accordance with mendelian principles, this allele was the one inherited from the mother. The loss of this allele is consistent with the occurrence of a submicroscopic deletion (at the level of banding we were able to achieve) in the short arm of chromosome 11 as a result of an 11p/12q translocation. This conclusion is supported by chromosome *in situ* hybridization studies¹⁵ which clearly demonstrate c-Ha-ras1 hemizygosity as the gene is not present on the derivative chromosome 11 or 12 but is on the intact chromosome 11.

Hybridization of the c-Ha-ras1 probe to Southern blots of *Bam*HI-digested DNA from direct tissue samples of tumour 2

gave a pattern similar to that obtained for cultured cells from tumour 1 (Fig. 2c). Leukocyte DNA contained two c-Ha-ras1 alleles (Fig. 2c, lane 4), only one of which was present in the tumour (lane 3). The father and mother were both apparently homozygous for the c-Ha-ras1 allele (lanes 1 and 2 respectively), and it is the maternally-derived allele which is absent from the tumour. As truncated *Bam*HI fragments were not observed in either of these tumours, it seems that extensive breakage or rearrangement of the c-Ha-ras1 gene had not occurred. This conclusion is substantiated by the observation that DNA from both tumour 1 and normal individuals contained a major large *Eco*RI fragment (tumour 2 was not analysed) hybridizing to the

c-Ha-ras1 probe (Fig. 2a, lanes 1, 3, 5, 7, 9 and 11). The c-Ha-ras1 gene has previously been mapped within this large fragment¹⁶. In addition to the two cases described above, two other Wilms' tumours have been analysed, but these were uninformative as the constitutional tissues were homozygous for c-Ha-ras1.

The data from Wilms' tumour 1 described in this study exemplify a mitotic mechanism by which hemizyosity of an allele may be achieved: the occurrence of a translocation associated with a loss of chromosomal material. This work extends the recent study by Cavenee *et al.*¹⁷ which demonstrated that in retinoblastoma, homozygosity or hemizyosity for a mutant allele may arise by mitotic recombination, nondisjunction, or other chromosomal rearrangements. The c-Ha-ras1 gene should be useful, therefore, as a genetic marker for chromosome changes in Wilms' tumour, analogous to the esterase marker and restriction fragment length polymorphism probes in retinoblastoma^{18,19}. Evidence supporting the notion that the Wilms' tumour and the c-Ha-ras1 genes are rather distant from each other has been adduced by two groups of investigators. Huerre *et al.*¹⁰ studied four subjects with a constitutional 11p deletion and aniridia, one of whom had a Wilms' tumour. Two were unequivocally constitutionally heterozygous for the BamHI-defined c-Ha-ras1 polymorphism, and two were interpreted on the basis of hybridization intensities to be homozygous. Tumour tissue from the single case with a Wilms' tumour showed that the "c-Ha-ras1 pattern was normal". De Martinville and Francke have reported the case of a girl with aniridia-Wilms' tumour association having a constitutional 11p12-11p14 deletion, who unequivocally possessed two c-Ha-ras1 alleles; and a remarkable family with individuals having one, two and three copies of the 11p11.2 to 11p14.1 region who all possessed two alleles⁹. In addition, the consensus of a recent gene mapping meeting placed c-Ha-ras1 at 11p15 (ref. 11). The c-Ha-ras1 allele loss that we have demonstrated in sporadic Wilms' tumour may therefore reflect events simultaneously affecting the Wilms' tumour locus and the quite distinct, distally placed c-Ha-ras1 gene. On the other hand, Jhanwar *et al.*⁸ have, by using *in situ* hybridization to meiotic chromosomes, localized c-Ha-ras1 to 11p14.1, immediately adjacent to the translocation break point in our tumour 1 (11p13). Thus, until the chromosomal location of c-Ha-ras1 has been determined with certainty, we cannot exclude a possible functional involvement of c-Ha-ras1 in Wilms' tumour development.

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Somatic deletion and duplication of genes on chromosome 11 in Wilms' tumours

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One of the most provocative findings in tumour biology is the relationship between chromosomal changes and embryonal cancers in children. For example, children with the rare paediatric syndrome AGR triad (aniridia, genito-urinary abnormalities and mental retardation) often develop Wilms' tumours at a very early age¹ and carry a germ-line deletion on the short arm of chromosome 11 (11p13)². It has been suggested that the germ-line deletion 11p is the first of two or more steps to cancer in AGR children². If this were true, one might expect a similar deletion to arise somatically in the far more common isolated Wilms' tumours of children without AGR, as suggested by Knudson³ from epidemiological data. However, a chromosomal deletion on 11p was observed in only two of five such cases^{4,5}, while it was absent or seen inconsistently in others^{5,6}. We have now used a molecular genetic approach to determine whether Wilms' tumour cells possess somatic alterations at 11p loci. We have found somatic deletions of specific genes in four of six Wilms' tumours. Surprisingly, in all four cases, the deletions were associated with duplications leading to homozygosity of the non-deleted alleles in the tumour cells. As analogous observations were recently reported in retinoblastoma⁷, the genetic events reported here may underlie the development of many such embryonal tumours in children.

The patients studied were aged between 1 and 9 years old and had histologically confirmed Wilms' tumours without the AGR triad. We compared Wilms' tumour DNA and normal kidney DNA at four loci on 11p: γ -globin, insulin, c-Ha-ras1 and parathyroid hormone (PTH). γ -Globin, insulin and c-Ha-ras1 have all been assigned to the same distal region of 11p (11p15-pter)⁸⁻¹¹, although c-Ha-ras1 has been localized more proximally by one group¹². In addition, PTH has been similarly assigned to 11p (ref. 13). The probable order, determined by linkage analysis, is PTH-globin-ras-insulin, spanning approximately 18 centimorgans (40% of 11p)¹⁴.

To determine the presence or absence of specific alleles, we exploited known DNA polymorphisms at these loci. These polymorphisms are normal inherited variations in DNA that mark the maternal and paternal copies of the affected gene. The variations can be detected by Southern blotting of the patient's DNA using a cloned copy of the gene of interest as a probe. By using this technique, we could determine unambiguously the presence, absence and copy number of individual alleles.

The technique is illustrated by our analysis of the γ -globin locus. We used a probe that detected both the γ -globin gene and highly related γ -globin gene; both genes display restriction site polymorphisms at a HindIII site localized within the second intron¹⁵. All six patients studied were heterozygous for a γ -globin gene polymorphism: for example, analysis of normal kidney DNA of patient 1 revealed two γ -globin alleles after HindIII digestion (alleles *a* and *b* in Fig. 1A). No γ -globin gene deletion was observed in tumour cells of this patient. In contrast, patient 2 was polymorphic for the γ -globin gene, revealing two γ -globin alleles (denoted *c* and *d* in Fig. 1A). In tumour tissue from patient 2, allele *c* was absent and thus had been deleted in somatic cells during the development of the Wilms' tumour. Patient 3 was polymorphic for both the γ -globin and γ -globin genes. DNA from the tumour of patient 3 showed the loss of one γ -globin allele (*a* in Fig. 1A) and one γ -globin allele (*c* in Fig. 1A; based on linkage data, the alleles are on the same chromosome¹⁶). Patient 4, while informa-

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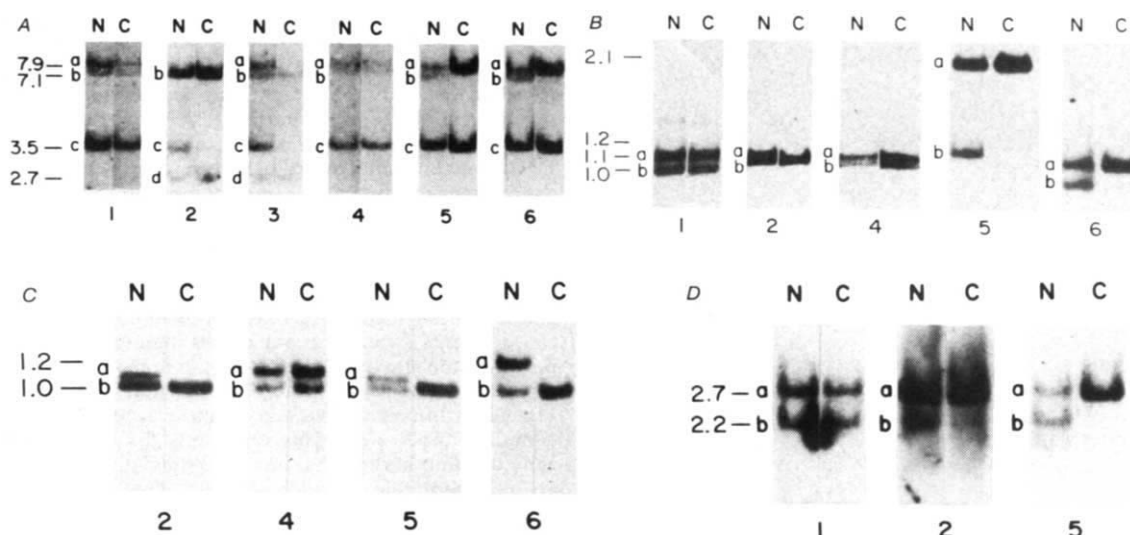


Fig. 1 Southern hybridization of 11p gene probes to normal kidney (N) and Wilms' tumour (C) DNA. Patient numbers are shown below the blots, and sizes in kilobase pairs (kbp) of the polymorphic bands (letters correspond to text and Table 1) are shown on the left. Only the informative blots (heterozygous for the polymorphism in the normal DNA) are shown. **A**, *HindIII* digest, γ -globin gene probe; **B**, *HinfI* digest, insulin gene probe; **C**, *MspI* digest, c-Ha-ras1 gene probe; **D**, *PstI* and *HindIII* digest, parathyroid hormone gene probe.

Methods: High-molecular weight DNA was prepared from minced Wilms' tumours and normal kidney tissues of the six patients. The specimens were pulverized in liquid nitrogen, digested overnight in SDS and proteinase K at 42 °C, sheared by passage through a 19-gauge needle, and extracted with phenol and chloroform as described elsewhere²⁸. DNA concentrations were determined by a diphenylamine assay²⁹. DNA (5 μ g) was digested with restriction enzyme, and the resulting DNA fragments were separated by electrophoresis through agarose gels and transferred to nitrocellulose filters as described^{30,31}. The following gene fragment probes were used: (1) a 1.1-kb *TaqI* cDNA fragment derived from the recombinant plasmid JW151 containing human γ -globin sequences³²; (2) a 1.2-kb *HinfI* genomic DNA fragment 5' of the human insulin gene¹⁸; (3) a 6.6-kb *BamHI* fragment derived from recombinant plasmid pEJ containing the entire c-Ha-ras1 gene³³, and a 0.99-kb *MspI* c-Ha-ras1 subfragment containing the polymorphic 3' flanking region; and (4) a 0.8-kb *HpaII* cDNA fragment derived from the recombinant plasmid pPTHm122 and containing human PTH gene sequences³⁴. The gene probe fragments were separated by electrophoresis and 'oligo-labelled' directly in the gel to a specific activity of $>10^9$ d.p.m. μ g⁻¹, as described previously^{35,36}. Southern hybridization³⁰, post-hybridization washing³⁷ and autoradiography³⁸ were performed as described elsewhere.

tive (heterozygous) at the γ -globin locus, showed no deletion in the tumour cells. However, both patients 5 and 6 exhibited somatic deletions of the γ -globin gene in the tumour cells (Fig. 1A). Hence, four of six patients developed a deletion of a γ -globin gene in their Wilms' tumours that was not manifest in their normal kidney tissue.

The human insulin gene often contains an insertion of variable length, located approximately 1 kilobase (kb) 5' from the start site of transcription^{17,18}. By using high-percentage agarose gels to resolve small differences in insertion size, and with a genomic probe spanning the insertion site, we were able to detect heterozygosity for this polymorphism in the normal cells of five of the six patients. Patients 1 and 4 retained both copies of the insulin gene in their tumour cells (Fig. 1B). However, patients 2, 5 and 6 all lost one allele of the insulin gene in their tumours, as shown by the loss in the tumour lanes of a 1.1-, 1.2-, and 1.0-kb band, respectively.

Like the insulin gene, c-Ha-ras1, the human homologue of the transforming gene of the Harvey murine sarcoma virus, displays a length polymorphism due to the presence of a small tandemly repeated sequence^{19,20}. By using high-percentage

agarose gels, we detected heterozygosity for this polymorphism in normal cells from four of the six patients. Patients 2, 5 and 6 showed loss of one allele of the c-Ha-ras1 gene in their tumours, while patient 4 retained both copies of the gene (Fig. 1C).

We used a *PstI* restriction site polymorphism located in exon 3 of the PTH gene to examine for deletions at this locus in the tumours²¹. Patients 1, 2 and 5 were informative at this locus, each demonstrating two bands in the Southern blots of their normal kidney DNA. While patient 1 exhibited no somatic deletion (as with other informative 11p genes in this patient), patients 2 and 5 had each lost one allele in their tumour cells (Fig. 1D).

In the patients displaying a deletion of a restriction fragment on Southern analysis, the remaining band appeared more intense than the corresponding band in the normal kidney tissue. To quantitate this observation, we rehybridized at least one filter from each of the four patients to a nonsynthetic probe derived from the human growth hormone (HGH) gene (on chromosome 17)²², or from the α -human chorionic gonadotropin (α -HCG) gene (on chromosome 18 (ref. 23) or chromosome 6 (ref. 24)).

Table 1 11p Alleles in normal kidney and Wilms' tumour tissues

Locus	Patient 1		Patient 2		Patient 3		Patient 4		Patient 5		Patient 6	
	Normal	Cancer	Normal	Cancer	Normal	Cancer	Normal	Cancer	Normal	Cancer	Normal	Cancer
γ -globin	a/b	a/b	b/b	b/b	a/b	b/b	a/b	a/b	a/b	a/a	a/b	a/a
γ -globin	c/c	c/c	c/d	d/d	c/d	d/d	c/c	c/c	c/c	c/c	c/c	c/c
Insulin	a/b	a/b	a/b	b/b	a/a	a/a	a/b	a/b	a/b	a/a	a/b	a/a
c-Ha-ras1	a/a	a/a	a/b	b/b	a/a	a/a	a/b	a/b	a/b	b/b	a/b	b/b
Parathyroid hormone	a/b	a/b	a/b	a/a	b/b	b/b	b/b	b/b	a/b	a/a	a/a	a/a

Allelic assignments were determined by Southern hybridization to 11p gene probes, as described in Fig. 1 legend, and in some cases by rehybridization to nonsynthetic genes and densitometric analysis, as described in Fig. 2 legend. The alleles are named as in Fig. 1.

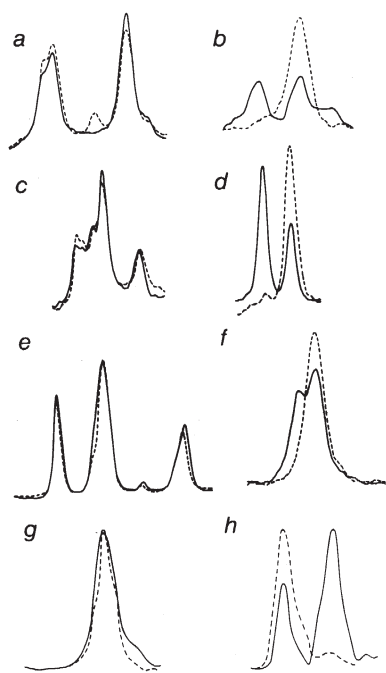


Fig. 2 Densitometric scans of Southern hybridizations with 11p gene probes and of rehybridizations with nonsyntenic genes. Solid lines, normal lanes; broken lines, tumour lanes. *a, b*, Patient 5, HGH and parathyroid hormone, respectively; *c, d*, patient 6, HGH and c-Ha-ras1; *e, f*, patient 2, HGH and insulin; *g, h*, patient 3, α -HCG and α -globin.

Methods: Southern blots (shown in Fig. 1) as well as other blots were freed of probe in alkali and rehybridized with either a 0.8-kb *Hind*III cDNA fragment derived from the recombinant plasmid chGH800/pBR322 containing HGH coding sequences³⁹, or a 0.6-kb cDNA fragment derived from the recombinant plasmid α -HCG/pBR322 containing the coding region of α -HCG⁴⁰. Grain density was quantitated by densitometric tracing using a Clifford Densicom model 445 densitometer. After normalizing the HGH and α -HCG scans to control for DNA loading, the 11p gene blots were rescanned to produce the tracings shown.

Autoradiograms of these blots were scanned with a densitometer and compared to determine the ratios of hybridization of restriction fragments in the normal and tumour DNA in the two blots. Two facts are evident from this analysis. First, the tumours from patients 2, 3, 5 and 6 had each lost an allele in over 95% of the cells from their tumours. The small amount of hybridization to the missing allele which persisted in the tumour DNA samples was probably due to the normal cells (connective and vascular tissue) within the Wilms' tumour samples (see Fig. 2). Second, in each of the four patients with deletions, the remaining 11p alleles were of approximately double intensity in the tumour cells (Fig. 2).

The alterations we observed (summarized in Table 1) could have involved most or all of chromosomal arm 11p or an entire chromosome 11. Despite intensive effort, none of the tumours studied here would propagate in culture to provide metaphase karyotypes. To confirm that the changes we observed were specific to 11p and not widespread random alterations, we also examined DNA polymorphisms at the human growth hormone gene (chromosome 17)²⁵ and the antithrombin III gene (chromosome 1)²⁶; no qualitative or quantitative changes were detected in the five informative patients (data not shown).

It is of interest to compare our results with those recently obtained from molecular analyses of other patient populations. Orkin *et al.* have observed somatic deletion-duplication of chromosome 11 alleles in one of seven Wilms' tumours from non-AGR triad patients (see the accompanying paper²⁷). Two recent studies have shown neither germ-line deletion of β -globin, c-Ha-ras1 or insulin genes in normal lymphocytes of five

individuals with the AGR triad and an interstitial germ-line deletion involving 11p13 (refs 8, 9), nor somatic deletion of β -globin or c-Ha-ras1 in one Wilms' tumour⁹. These results, together with those presented here, suggest heterogeneity in the mechanism of Wilms' tumorigenesis.

In a study of retinoblastoma, another embryonal tumour, DNA sequence polymorphisms on chromosome 13 demonstrated gene deletion in five of eight tumours, often with duplication of the non-deleted alleles⁷. Thus, deletion-duplication may be involved generally in development of embryonal tumours.

What is the biological significance of our observation of gene duplication in Wilms' tumour? There are at least two possibilities: (1) mitotic recombination or nondisjunction-duplication events (which cannot be distinguished without further polymorphic markers), might result in homozygosity for a recessive tumorigenic allele; and (2) a deletion of a large region of 11p might impair cell viability, unless accompanied by a concomitant duplication. This represents the first observation of gene deletion and duplication in Wilms' tumours and suggests an important role for such somatic genetic alterations in the biology of this cancer.

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Are U4 small nuclear ribonucleoproteins involved in polyadenylation?

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The mechanism whereby eukaryotic pre-messenger RNAs are polyadenylated is unknown. Most models for polyadenylation invoke cleavage of precursor transcripts at the site of poly(A) addition followed by polymerization of A residues by poly(A) polymerase¹⁻⁵. Analysis of the sequences surrounding poly(A) addition sites has identified the consensus recognition sequence element AAUAAA as necessary but not sufficient for polyadenylation⁶⁻¹⁴. A second recognition sequence element CACUG, was observed by Benoit *et al.*¹⁵ to be adjacent to the site of poly(A) addition in several sequenced RNAs. Here, we analyse 61 vertebrate poly(A) addition sequences, define a more extensive recognition sequence for polyadenylation than previously recognized and suggest how the site of poly(A) addition may be chosen. Furthermore, we find that the defined recognition sequence has elements which are complementary to regions within the small nuclear RNA U4, suggesting that U4 small nuclear ribonucleoproteins (snRNPs) may mediate polyadenylation in a fashion similar to the role of U1 snRNPs in splicing. The model invokes hybridization of U4 RNA to AAUAAA recognition elements as related to primary site selection, and hybridization to CAYUG recognition elements as related to cleavage site selection.

Sequences of pre-polyadenylated RNAs were extrapolated from available genomic DNA sequences. The sequences were grouped into two classes exemplified by the simian virus 40 (SV40) early (class I) and late (class II) RNAs (Fig. 1). The two classes were distinguished by the differential location of CAYUG recognition elements upstream or downstream, respectively, from the site of poly(A) addition. SV40 early prepolyadenylated RNA contains a copy of the pentanucleotide CACUG immediately upstream from the site of poly(A) addition. The SV40 late RNA contains three abutted copies of versions of the related sequence CAUUG located immediately downstream from the site of poly(A) addition.

Table 1 catalogues the sequences surrounding the sites of poly(A) addition for the 61 RNAs examined. The table groups the analysed sequences into the two classes exemplified by the SV40 early and late RNAs. The previously recognized hexanucleotide AAUAAA was clearly the dominant feature of all 61 polyadenylation sites. The sequence was highly conserved, only a few RNAs containing shortened versions of this sequence; no substitutions other than AUUAAA were observed. The sequence was repeated in tandem in 30% of the examined sequences. The most downstream copy was an average of 15-17 nucleotides upstream from the site of poly(A) addition in both

classes of sequences, regardless of the presence of intervening recognition sequence elements.

A representative of the pentanucleotide sequence CACUG was present either upstream or downstream from the site of poly(A) addition in every RNA examined when the sequence was considered as CAYUG. Some RNAs had more than one copy of this sequence clustered around the poly(A) addition site; such repeats were more common in class II sequences in which the pentanucleotide was located downstream. Polyadenylation occurred at the nearest A to the observed pentanucleotide with the restriction that downstream UA sequences were rarely utilized. The sequence was neither as complete nor as conserved as the consensus AAUAAA sequence, which suggests that it is not the prominent feature by which the polyadenylation machinery selects polyadenylation sites. Instead, this sequence had properties more suggestive of a role in determining the specificity of cleavage within a pre-selected site. Recognition elements occurring downstream from the site of poly(A) addition have not previously been noted. Their downstream positioning strongly suggests that the cleavage event occurring during polyadenylation is located at the poly(A) addition site.

Because both polyadenylation and splicing involve endonucleolytic cleavage of precursor RNAs, the possibility was investigated that small nuclear RNAs could be involved in polyadenylation as well. Therefore, U1, U2, U4, U5 and U6 RNAs were examined for sequences which could hybridize to portions of the polyadenylation sites described above^{16,17}. Only U4 RNA contained a pentanucleotide sequence complementary to AAUAAA or AAUUAU. Furthermore, U4 possesses three such sequences in close proximity (Fig. 2; two would hybridize to AAUAAA, one to AAUUAU). Only U4 RNA contained a pentanucleotide complementary to the CAYUG recognition element. Furthermore, U4 contained two such sequences and both were located upstream from the three sequences complementary to AAUAAA. The two U4 pentanucleotides varied in that one would hybridize to CACUG recognition elements whereas the other would recognize CAUUG elements. The 5' to 3' polarity of the two blocks of sequences within U4 RNA would permit simultaneous hybridization to the two blocks of recognition elements within a pre-polyadenylated RNA. Furthermore, multiple copies of each complementary sequence block within U4 RNA would permit considerable potential for hybridization with those pre-polyadenylated RNAs possessing multiple copies of the individual recognition elements. Figure 3 outlines possible interactions between U4 RNA and the two SV40 polyadenylation sites shown in Fig. 1.

The five complementary pentanucleotides in U4 RNA reside within the 5'-terminal 70 nucleotides. This is a region of U4 RNA thought not to include protein recognition information for U4 snRNP assembly, and it might, therefore, be available to interact with pre-polyadenylated RNA^{18,19}. The U4 RNA secondary structure shown in Fig. 2 was determined for isolated U4 RNA rather than for U4 RNA in snRNPs. Thus, the actual *in vivo* secondary structure of U4 RNA is unclear. Furthermore,

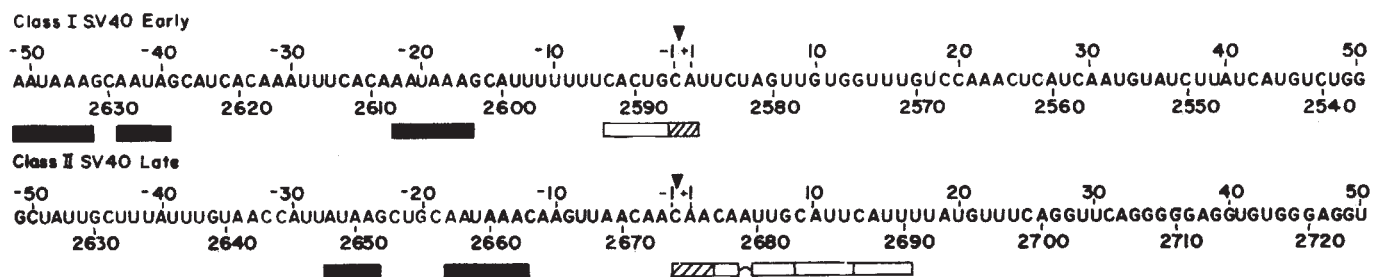


Fig. 1 Sequences surrounding polyadenylation sites for SV40 early and late prepolyadenylated RNAs. Numbering above the sequence is with respect to the nucleotide at which poly(A) addition occurs^{11,12}; the first A of the poly(A) addition site is numbered +1, the nucleotide immediately upstream is denoted -1. SV40 nucleotide numbers are indicated beneath the sequences. The three primary sequence elements scored for each RNA in this survey are underlined for these two examples of class I (early) and class II (late) polyadenylation sites. Solid bars, consensus AAUAAA sequences; open bars, CACUG sequences; and hatched bars, poly(A) addition sites.

Table 1 Polyadenylation sites

CLASS I				CLASS II			
AAUAAA 11	AAUAAA 16	CAC	6 CAA	AAUAAA 13	CU	6 ACUG	CHICKEN INSULIN
AAUAAA 10	CAUUG	6 CAA		AAUAAA 12	CAAA	CACU	5 CAUU
AAUAAA 13	CACU	CA		AAUAAA 9	CAA	CACU	1 CACUG
AAUAAA	CACUG	CA UG	CA	AAUAAA 10	CAAA	C CUG	C UUG
AAUAAA 6	CAUUG	C CUG	A	AAUAAA 10	UAA	1 CAUU	CHICKEN OVOMUCOID
AAUAAA 6	CAUUG	5 GA		AAUAAA 19	GAA	2 C CUG	5 C CUG
AAUAAA 11	C UUG	CAA		AAUAAA 11	CAAAA	CA UG	1 CAUU
AAUAAA 13	CAUUG	CAA		AAUAAA 14	CA	1 C CUG	
AAUAAA 12	C UUG	4 GAA		AAUAAA 12	CAA	C CUG	5 CA
AAUAAA 10	AAUAAA 9	ACUG	A	AAUAAA 13	UAA	CAUUG	CAUU
AAUAAA 17	AAUAAA 8	CAUU	AAAA	AAUAAA 14	UAA	C CUG	4 C UUG
AAUAAA 20	C UU	UA		AAUAAA 15	UAA	C UUG	CA UG
AAUAAA 8	CAU	CAAAA		AAUAAA 16	UAA	CAUUG	CAUU
AAUAAA 17	C CUG	CA		AAUAAA 17	UAA	CAUUG	CAUU
AAUAAA 15	CACUG	CAA		AAUAAA 18	UAA	CAUUG	CAUU
AAUAAA 26	CACU	2 CA		AAUAAA 19	UAA	CAUUG	CAUU
AAUAAA 7	CACUG	1 GA		AAUAAA 20	UAA	CAUUG	CAUU
AAUAAA 8	CACUG	C UUG	1 GA	AAUAAA 21	UAA	CAUUG	CAUU
AAUAAA 12	C CUG	1 GA		AAUAAA 22	UAA	CAUUG	CAUU
AAUAAA 11	CACU	1 GA		AAUAAA 23	UAA	CAUUG	CAUU
AAUAAA 13	CAUUG	CAA		AAUAAA 24	UAA	CAUUG	CAUU
AAUAAA 11	CACUG	1 GA		AAUAAA 25	UAA	CAUUG	CAUU
AAUAAA 13	CACUG	1 GA		AAUAAA 26	UAA	CAUUG	CAUU
AAUAAA 13	CACUG	1 GA		AAUAAA 27	UAA	CAUUG	CAUU
AAUAAA 13	CACUG	1 GA		AAUAAA 28	UAA	CAUUG	CAUU
AAUAAA 13	CACUG	1 GA		AAUAAA 29	UAA	CAUUG	CAUU
AAUAAA 13	CACUG	1 GA		AAUAAA 30	UAA	CAUUG	CAUU
AAUAAA 13	CACUG	1 GA		AAUAAA 31	UAA	CAUUG	CAUU
AAUAAA 13	CACUG	1 GA		AAUAAA 32	UAA	CAUUG	CAUU
AAUAAA 13	CACUG	1 GA		AAUAAA 33	UAA	CAUUG	CAUU
AAUAAA 13	CACUG	1 GA		AAUAAA 34	UAA	CAUUG	CAUU
AAUAAA 13	CACUG	1 GA		AAUAAA 35	UAA	CAUUG	CAUU
AAUAAA 13	CACUG	1 GA		AAUAAA 36	UAA	CAUUG	CAUU
AAUAAA 13	CACUG	1 GA		AAUAAA 37	UAA	CAUUG	CAUU
AAUAAA 13	CACUG	1 GA		AAUAAA 38	UAA	CAUUG	CAUU
AAUAAA 13	CACUG	1 GA		AAUAAA 39	UAA	CAUUG	CAUU
AAUAAA 13	CACUG	1 GA		AAUAAA 40	UAA	CAUUG	CAUU
AAUAAA 13	CACUG	1 GA		AAUAAA 41	UAA	CAUUG	CAUU
AAUAAA 13	CACUG	1 GA		AAUAAA 42	UAA	CAUUG	CAUU
AAUAAA 13	CACUG	1 GA		AAUAAA 43	UAA	CAUUG	CAUU
AAUAAA 13	CACUG	1 GA		AAUAAA 44	UAA	CAUUG	CAUU
AAUAAA 13	CACUG	1 GA		AAUAAA 45	UAA	CAUUG	CAUU
AAUAAA 13	CACUG	1 GA		AAUAAA 46	UAA	CAUUG	CAUU
AAUAAA 13	CACUG	1 GA		AAUAAA 47	UAA	CAUUG	CAUU
AAUAAA 13	CACUG	1 GA		AAUAAA 48	UAA	CAUUG	CAUU
AAUAAA 13	CACUG	1 GA		AAUAAA 49	UAA	CAUUG	CAUU
AAUAAA 13	CACUG	1 GA		AAUAAA 50	UAA	CAUUG	CAUU
AAUAAA 13	CACUG	1 GA		AAUAAA 51	UAA	CAUUG	CAUU
AAUAAA 13	CACUG	1 GA		AAUAAA 52	UAA	CAUUG	CAUU
AAUAAA 13	CACUG	1 GA		AAUAAA 53	UAA	CAUUG	CAUU
AAUAAA 13	CACUG	1 GA		AAUAAA 54	UAA	CAUUG	CAUU
AAUAAA 13	CACUG	1 GA		AAUAAA 55	UAA	CAUUG	CAUU
AAUAAA 13	CACUG	1 GA		AAUAAA 56	UAA	CAUUG	CAUU
AAUAAA 13	CACUG	1 GA		AAUAAA 57	UAA	CAUUG	CAUU
AAUAAA 13	CACUG	1 GA		AAUAAA 58	UAA	CAUUG	CAUU
AAUAAA 13	CACUG	1 GA		AAUAAA 59	UAA	CAUUG	CAUU
AAUAAA 13	CACUG	1 GA		AAUAAA 60	UAA	CAUUG	CAUU
AAUAAA 13	CACUG	1 GA		AAUAAA 61	UAA	CAUUG	CAUU
AAUAAA 13	CACUG	1 GA		AAUAAA 62	UAA	CAUUG	CAUU
AAUAAA 13	CACUG	1 GA		AAUAAA 63	UAA	CAUUG	CAUU
AAUAAA 13	CACUG	1 GA		AAUAAA 64	UAA	CAUUG	CAUU
AAUAAA 13	CACUG	1 GA		AAUAAA 65	UAA	CAUUG	CAUU
AAUAAA 13	CACUG	1 GA		AAUAAA 66	UAA	CAUUG	CAUU
AAUAAA 13	CACUG	1 GA		AAUAAA 67	UAA	CAUUG	CAUU
AAUAAA 13	CACUG	1 GA		AAUAAA 68	UAA	CAUUG	CAUU
AAUAAA 13	CACUG	1 GA		AAUAAA 69	UAA	CAUUG	CAUU
AAUAAA 13	CACUG	1 GA		AAUAAA 70	UAA	CAUUG	CAUU
AAUAAA 13	CACUG	1 GA		AAUAAA 71	UAA	CAUUG	CAUU
AAUAAA 13	CACUG	1 GA		AAUAAA 72	UAA	CAUUG	CAUU
AAUAAA 13	CACUG	1 GA		AAUAAA 73	UAA	CAUUG	CAUU
AAUAAA 13	CACUG	1 GA		AAUAAA 74	UAA	CAUUG	CAUU
AAUAAA 13	CACUG	1 GA		AAUAAA 75	UAA	CAUUG	CAUU
AAUAAA 13	CACUG	1 GA		AAUAAA 76	UAA	CAUUG	CAUU
AAUAAA 13	CACUG	1 GA		AAUAAA 77	UAA	CAUUG	CAUU
AAUAAA 13	CACUG	1 GA		AAUAAA 78	UAA	CAUUG	CAUU
AAUAAA 13	CACUG	1 GA		AAUAAA 79	UAA	CAUUG	CAUU
AAUAAA 13	CACUG	1 GA		AAUAAA 80	UAA	CAUUG	CAUU
AAUAAA 13	CACUG	1 GA		AAUAAA 81	UAA	CAUUG	CAUU
AAUAAA 13	CACUG	1 GA		AAUAAA 82	UAA	CAUUG	CAUU
AAUAAA 13	CACUG	1 GA		AAUAAA 83	UAA	CAUUG	CAUU
AAUAAA 13	CACUG	1 GA		AAUAAA 84	UAA	CAUUG	CAUU
AAUAAA 13	CACUG	1 GA		AAUAAA 85	UAA	CAUUG	CAUU
AAUAAA 13	CACUG	1 GA		AAUAAA 86	UAA	CAUUG	CAUU
AAUAAA 13	CACUG	1 GA		AAUAAA 87	UAA	CAUUG	CAUU
AAUAAA 13	CACUG	1 GA		AAUAAA 88	UAA	CAUUG	CAUU
AAUAAA 13	CACUG	1 GA		AAUAAA 89	UAA	CAUUG	CAUU
AAUAAA 13	CACUG	1 GA		AAUAAA 90	UAA	CAUUG	CAUU
AAUAAA 13	CACUG	1 GA		AAUAAA 91	UAA	CAUUG	CAUU
AAUAAA 13	CACUG	1 GA		AAUAAA 92	UAA	CAUUG	CAUU
AAUAAA 13	CACUG	1 GA		AAUAAA 93	UAA	CAUUG	CAUU
AAUAAA 13	CACUG	1 GA		AAUAAA 94	UAA	CAUUG	CAUU
AAUAAA 13	CACUG	1 GA		AAUAAA 95	UAA	CAUUG	CAUU
AAUAAA 13	CACUG	1 GA		AAUAAA 96	UAA	CAUUG	CAUU
AAUAAA 13	CACUG	1 GA		AAUAAA 97	UAA	CAUUG	CAUU
AAUAAA 13	CACUG	1 GA		AAUAAA 98	UAA	CAUUG	CAUU
AAUAAA 13	CACUG	1 GA		AAUAAA 99	UAA	CAUUG	CAUU
AAUAAA 13	CACUG	1 GA		AAUAAA 100	UAA	CAUUG	CAUU

Each sequence was examined for the presence of possible consensus recognition sequence elements surrounding poly(A) addition sites. The analysis only included examples for which downstream sequences were available. Because the model for polyadenylation derived from this analysis invokes hybridization to U4 RNA, only RNA sequences from organisms closely related to those for which a U4 RNA sequence was available were examined. This limited the analysis to 61 vertebrate sequences from chicken, mouse, rat, rabbit and man. Each sequence was scanned for three structural features: (1) the consensus recognition sequence AAUAAA (or AUUAAA) and its relative location with respect to the site of poly(A) addition; (2) the pentanucleotide sequence CACUG (or a close derivative, CAUUG) and its relative location with respect to the site of poly(A) addition; and (3) the sequence at the site of poly(A) addition. Sequences are aligned by their poly(A) addition sites (arrow). This alignment was to the N of the NAAA usually seen at this site; actual cleavage could occur at this nucleotide or at any of the succeeding A residues. Sequences for which the poly(A) addition site were putative were aligned by best-fit to the consensus sequence. Intervening nucleotides between individual elements of the consensus sequences are indicated numerically. Intervening or missing nucleotides in an individual element are shown as inserts above the line or gaps, respectively. A derived consensus sequence including percentage fit is shown for each class of sites at the bottom of the table. Single intervening nucleotides were permitted and were scored as detracting half from each neighbouring nucleotide. Nonreferenced sequences are contained in the May 1983 Genbank listing. (Genbank is the Genetic sequence data bank established by Bott Beranek and Newman Inc. and Los Alamos National Laboratory under contract with the NIH.) DHFR, dihydrofolate reductase; POMC, proopiomelanocortin.

* Ref. 30.

because U4 and U6 RNAs appear to reside in a single snRNP assembly²⁰ (K. Hashimoto and J. A. Steitz, personal communication), the conformation of U4 RNA and the interaction of U4 RNA with polyadenylation sites may be influenced by hybridization to U6 RNA.

Fitzgerald and Shenk have constructed a collection of mutants within the polyadenylation site for SV40 late RNA⁷. The properties of their deletions and insertions are consistent with the model described here. Deletions between the AAUAAA element and the poly(A) addition site moved the positions of poly(A) addition further downstream. By the definitions of the model described here, SV40 late RNA contains a class II site in which multiple CAUUG sequences are downstream from the poly(A) addition site and outside the region of the constructed deletions. Furthermore, because the CAUUG sequence is repeated three times in the SV40 late RNA sequence, considerable deletion should be possible before polyadenylation is abolished. Two mutants were generated in which alternative sequences were inserted downstream from the UUAUUU but before the poly(A) addition site. Both constructions not only introduced an acceptable NAA to act as a poly(A) addition site but also provided a reasonable downstream pentanucleotide CAYUG sequence (CACCUG in 1412 and CACU in 1411).

Recently, Montell *et al.*²¹ have isolated a point mutation within an AAUAAA sequence (a U → G transversion) which affected the efficiency of cleavage but not the specificity of cleavage or the addition of A residues. This result is consistent with the two-part recognition sequences for polyadenylation suggested here, an AAUAAA element required for primary selection of a sequence to be polyadenylated and a CAYUG element utilized to position the site of cleavage.

The postulated hybridization of polyadenylation sites to U4 RNA is analogous to the base-pairing thought to occur between U1 RNA and donor splice junctions^{22,23}. Of the five U RNAs, U1 and U4 appear most closely related at the sequence level^{16,17}. Comparison of the two RNA sequences indicates that the nucleotides in U1 RNA important for splice junction recognition are missing from U4 RNA. Similarly, the important regions of U4 RNA postulated here to interact with poly(A) addition sites are either missing from U1 RNA or contain internal base changes that would make hybridization to the polyadenylation site recognition elements very unfavourable. U4 RNA and U4 snRNPs are abundant in higher eukaryotes; a HeLa cell contains ~10⁶ U1 snRNPs and ~10⁵ U4 snRNPs. This relative abundance is consistent with the relative abundance of splice junctions to polyadenylation sites in pre-polyadenylated RNA and, there-

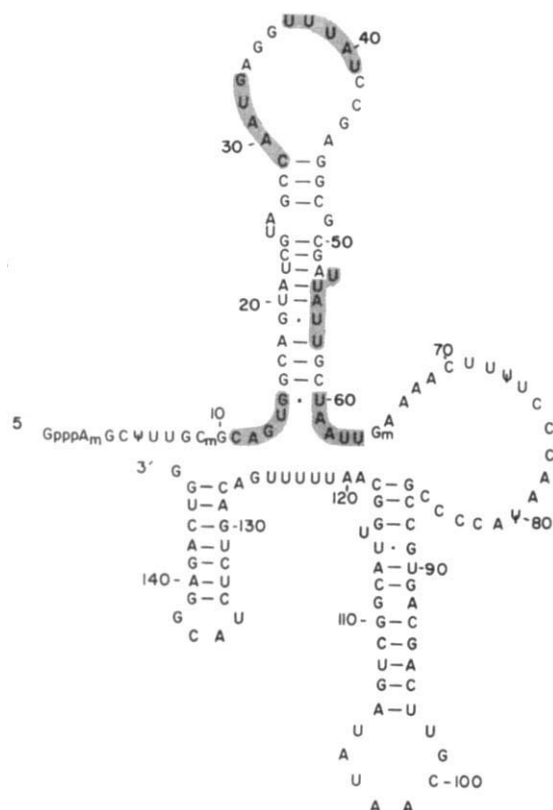


Fig. 2 One possible secondary structure of U4 RNA. The sequence shown is that of human U4 RNA¹⁷. Sequences postulated to hybridize to pre-polyadenylated RNA are indicated by shaded boxes.

fore, with the role for U4 RNA suggested here.

Several facets of polyadenylation cannot be explained by the present model. It might have been predicted that an adaptor RNA recognizing a polyadenylation site should have sequences capable of hybridizing to all six conserved nucleotides in the AAUAAA element rather than multiple pentanucleotide sequences. Point mutations in each of the nucleotides of the AAUAAA sequence may be required before the full function of this sequence can be ascertained. The model also does not fully explain why several unused AAUAAA sequences are bypassed by the polyadenylation machinery, given given the simplicity of the CAYUG sequence in several of the sequences in Table 1. Obviously, there are other requirements of polyadenylation which remained unexplained.

In yeast, the RNA snR3 exhibits homology to both U4 and U6 RNAs and may represent an evolutionary fusion of the two RNAs²⁴. The locus for snR3 is dispensable in yeast²⁵. The lack of requirement for a factor as evolutionarily conserved as one of the U RNAs is puzzling and led Tollervey *et al.* to postulate multiple factors carrying out the function of U4 and U6 RNAs in yeast²⁵. In light of the model for U4 function presented here, it is interesting that many polyadenylated yeast RNAs do not contain an AAUAAA sequence element.

The suggestion that more than one snRNP may be involved in RNA processing raises the intriguing possibility of snRNP-mediated communication between various portions of the RNA processing machinery. To date no enzymatic properties have been associated with snRNPs or their proteins. Instead, U1 snRNPs, which have been most extensively studied, have properties more suggestive of their being nucleic acid binding moieties than enzymes²⁶. In this context, snRNPs become the recognition factors that mediate interaction between primary sequences on RNA precursors and other elements of the processing machinery.

In vitro polyadenylation systems have recently been developed²⁷⁻²⁹. One of these systems has been shown to be

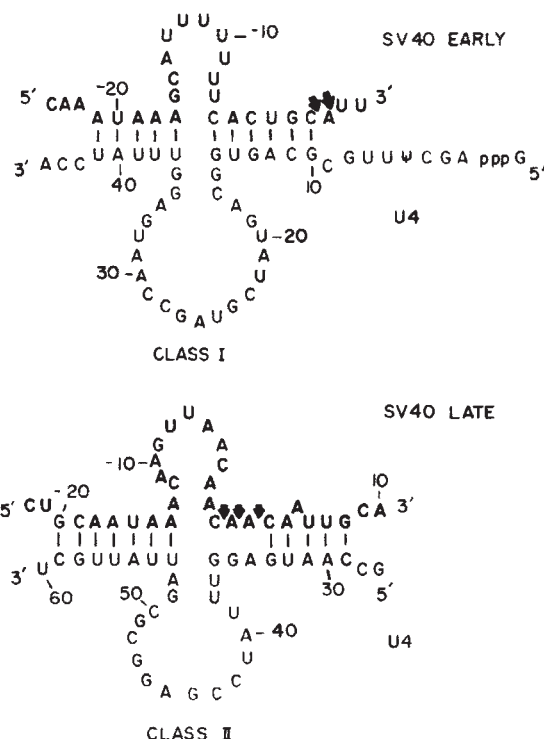


Fig. 3 Possible modes of hybridization between human U4 RNA and class I (SV40 early) and class II (SV40 late) polyadenylation sites. The class I hybridization utilizes U4 sequences at nucleotides 10-15 and 37-41. The class II hybridization utilizes U4 sequences at nucleotides 29-33 and 53-59. Other modes are also possible. Possible sites of poly(A) addition are indicated with arrows.

inhibited by a monoclonal anti-Sm antibody²⁹. This antibody recognizes all five U RNA snRNPs, indicating that one or more of these ribonucleoproteins is involved in polyadenylation in this system. Demonstration that the actual participating snRNP is that containing U4 RNA must await the development of U4-specific reagents.

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Monoclonal antibodies distinguish between storage and secreted forms of eosinophil cationic protein

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The toxic effects of eosinophils on parasites¹ and cells² are due largely to the secretion of various granule proteins, following stimulation³. In order to study this secretory process (degranulation) further, we have raised mouse monoclonal antibodies against both human eosinophil granule extracts and secretion products. From immunocytochemical studies it appears that one antibody, EG1, recognized both the storage and secreted forms of eosinophil cationic protein (ECP), whereas antibody EG2 only bound to ECP during secretion (and extraction). This antibody also bound to eosinophil protein-X (EP-X). As both antibodies stained eosinophils in formalin-fixed tissues, they were used to demonstrate sites of eosinophil activation and secretion in chronic urticaria. The capacity of monoclonal antibodies to detect differences between storage and secreted forms of proteins is an important property of these reagents with many potential applications in cell biology.

Human eosinophils contain several basic proteins including eosinophil major basic protein (MBP)⁴, ECP⁵, EP-X⁶ (which is possibly identical to eosinophil derived neurotoxin EDN)⁷, and eosinophil peroxidase (EPO)⁸. Rabbit antibodies have been raised to each of these proteins, and their concentrations have been measured in sera^{8–10}. Eosinophils are involved not only in host defence against parasites, especially nematodes¹¹, but may also damage tissues such as bronchial epithelium¹² and heart cells². More recently, immunofluorescence studies on fixed tissues have shown the localization of MBP in biopsies from patients with asthma¹³, and several types of skin disease¹⁴. The capacity of eosinophils to cause injury has now been shown to be increased by 'activation' in which the cells acquire an additional capacity to be cytotoxic to antibody-coated parasites^{15–17} and tumour cells *in vitro*¹⁸. Activated eosinophils have been found in the blood of some patients with an eosinophilia^{3,19}, and normal blood eosinophils have recently been shown to become activated after incubation with factors derived from monocytes, lymphocytes^{15,16} and tumour cells¹⁸. Although there are many examples of latent storage enzymes becoming active (including granulocyte collagenase²⁰), there are no precedents for the appearance of neoantigens on secreted proteins. However it was thought that the remarkable capacity of the strongly basic proteins in the eosinophil crystalloid granules to become solubilized was likely to be associated with alterations in their size, three-dimensional structure and/or antigenicity.

To investigate this, eosinophils were purified²¹ from the blood of patients with the hypereosinophilic syndrome²². Granule proteins were extracted from >90% pure eosinophils using 0.2 M acetate buffer, pH 4.2 (ref. 5) and secretion products were made by stimulating purified granulocytes (70% eosinophils,

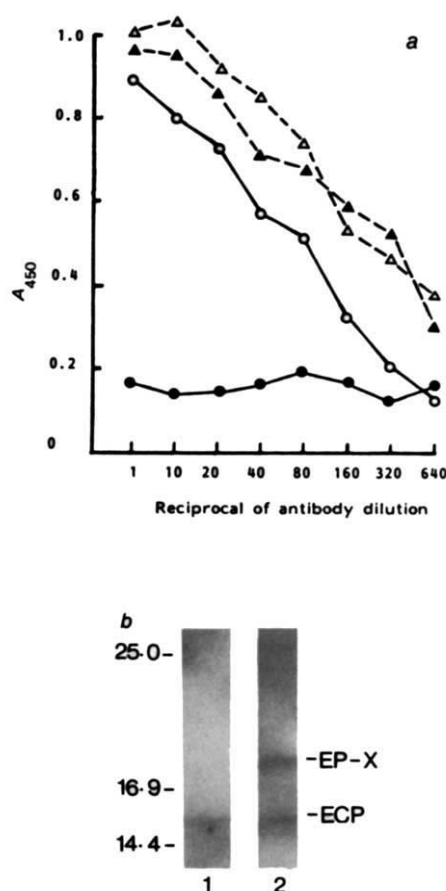


Fig. 1 *a*, Binding of monoclonal antibodies EG1 and 2 to purified ECP and EP-X. ○, antibody EG1 and ECP; ●, antibody EG1 and EP-X; △, antibody EG2 and ECP; and ▲, antibody EG2 and EP-X. Culture supernatants containing antibodies were serially diluted and added to wells of microtitre plates coated with either purified ECP or EP-X, 5 µg ml⁻¹ at 37 °C for 1 h. The amount of antibody binding to each well was measured at 450 nm with peroxidase-conjugated rabbit anti-mouse IgG and H₂O₂ with *o*-phenylenediamine. *b*, Western blots of purified eosinophil ECP and EP-X with antibodies EG1 and EG2 (ref. 29). Purified ECP and EP-X were run on 15% dissociating SDS-PAGE and transferred electrophoretically overnight to nitrocellulose paper. They were then reacted with antibodies EG1 or EG2, treated with ¹²⁵I labelled rabbit anti-mouse IgG, and washed. Autoradiographs of the gels were developed after 1 week. These showed that antibody EG1 (lane 1) bound to ECP, whereas antibody EG2 (lane 2) bound to both ECP and EP-X. Molecular weight markers run at the same time are indicated on the left of the figure.

30% neutrophils) from a patient with the hypereosinophilic syndrome, with zymosan-C3b (ref. 23). The supernatants were collected and stored at -70 °C. Lymphocytes from CBA × BALB/c mice, immunized with 100 µg of extracted or secreted proteins, were fused subsequently to the mouse myeloma cell line PS-NS1 AG4.1 (ref. 24). Hybridomas were tested for the production of antibodies to eosinophil granule proteins using an indirect enzyme-linked solid phase immunoassay, and positive cultures were cloned three times by limiting dilution. Two of these clones produced antibodies of the IgG1 subtype: EG1, derived from a mouse injected with eosinophil extracts, and EG2, derived from a mouse injected with eosinophil secretion products. The specificities of the monoclonal antibodies were assessed with a wide range of purified eosinophil and neutrophil granule proteins (Table 1).

Antibody EG1 was specific for ECP, while antibody EG2 bound to both purified ECP and EP-X. The binding capacity of these two antibodies for ECP and EP-X was also shown by

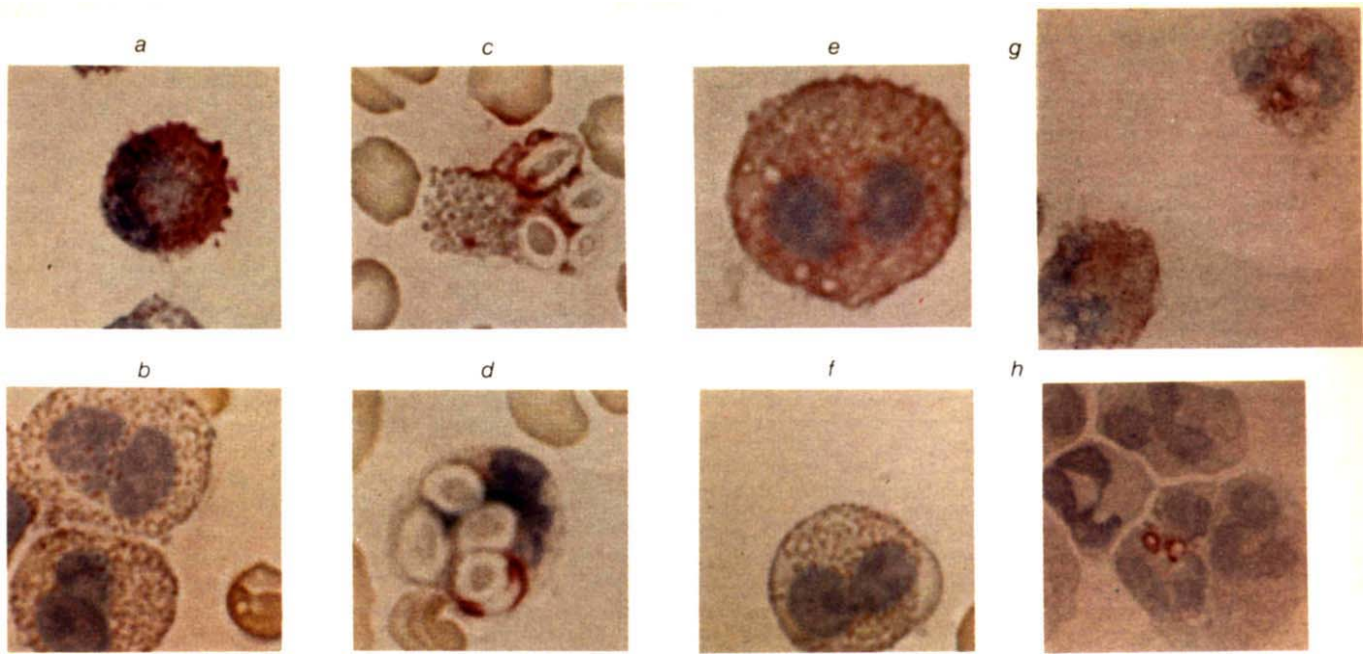


Fig. 2 *a, b*, Blood eosinophils from a normal individual reacted with antibodies EG1 and EG2 respectively. $\times 600$ and 750 . *c, d*, Eosinophils from a normal individual, after they had phagocytosed zymosan-C3b (ref. 23), and had been treated with antibodies EG1 and EG2 respectively. Zymosan-C3b was incubated with normal eosinophils for 1 h at 37°C , and cytocentrifuge preparations were made. $\times 700$. *e, f*, Normal eosinophils, after incubation with eosinophil cytotoxicity enhancing factor⁹ at 37°C for 1 h. Cytocentrifuge preparations of these cells were then treated either with antibody EG2 (*e*) or control antibody (*f*). $\times 750$ and 600 . *g, h*, Blood eosinophils from a patient with hypereosinophilic syndrome, treated with antibodies EG1 and EG2 respectively. $\times 600$.

Methods: Immunocytochemical staining of eosinophils with antibody EG1 (anti-ECP) and EG2 (anti-ECP, anti-EP-X). Blood eosinophils were isolated by centrifugation on discontinuous metrizamide density gradients²¹, and cytocentrifuge preparations were made. Slides were fixed with methanol, flooded with the antibody under study for 30 min and washed. Immunocytochemical staining was then carried out, using alkaline phosphatase-conjugated second antibodies^{30,31}. The alkaline phosphatase-conjugated rabbit anti-mouse IgG antibody was followed by alkaline phosphatase-conjugated goat anti-rabbit IgG antibody, naphthol AS-MX phosphate and Fast red with 1 mM levamisole to inhibit endogenous alkaline phosphatase. The red reaction product was allowed to develop for 30 min. The slides were then washed, counterstained with haematoxylin and mounted in Apathy's medium³⁰. Control slides were treated with an IgG1 mouse monoclonal anti-rat Thy-1 antibody (OX7, SeraLab) and stained in an identical fashion, to control for the specificity of the reaction on test slides. The mean % eosinophils stained is shown in each panel. Note that the slides were not treated with eosin.

Fig. 3 The localization of activated eosinophils and their secretion products in the dermis of a patient with chronic urticaria. Serial paraffin sections of the skin biopsy were dewaxed and rehydrated, and then stained with antibody EG2 as described in the legend to Fig. 2, and counterstained with haematoxylin. The red reaction product (Fast red) defines activated and secreting eosinophils, and the presence of secreted ECP and EP-X. *a*, Activated and secreting eosinophils were localized in the dermis. Several areas of extracellular deposits of ECP and EP-X were evident. $\times 300$. *b*, Control slide stained with an unrelated monoclonal antibody. $\times 300$.

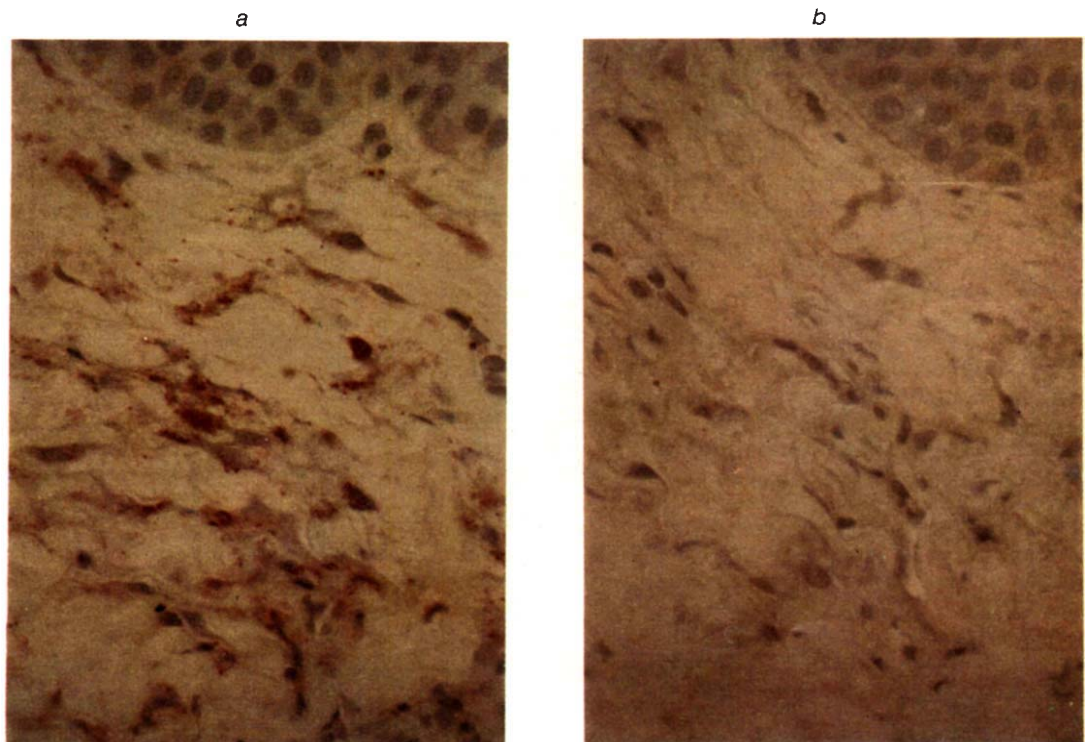


Table 1 Specificities of monoclonal antibodies EG1 and EG2 for human eosinophil granule proteins

Purified granule protein	Monoclonal antibodies	
	EG1	EG2
Eosinophil granule proteins	Optical density at 450 nm	
Eosinophil cationic protein, ECP	0.89*	1.02*
Eosinophil protein X, EP-X	0.16	0.95*
Major basic protein, MBP	0.09	0.06
Peroxidase, EPO	0.13	0.15
Neutrophil granule proteins		
Chymotrypsin-like cationic protein	0.08	0.06
Elastase	0.08	0.12
Lactoferrin	0.16	0.13
Myeloperoxidase	0.14	0.09

ECP was purified from blood granulocytes of a patient with chronic eosinophilic myeloid leukaemia⁵. EPO and EP-X were prepared from purified blood eosinophils of a patient with hypereosinophilia⁶. Their purity was assessed by agarose-gel electrophoresis, SDS-polyacrylamide gel electrophoresis (PAGE) of the reduced proteins, and double immunodiffusion in agar with specific polyclonal antibodies³. EPO was purified by Sephadex G-75 and CM-Sephadex column chromatography and identified by its enzyme activity. The purity of EPO was determined by comparison with an extinction value at 415 and 280 nm of 1.10, agarose-gel electrophoresis, SDS-PAGE after reduction, and by its failure to precipitate in double immunodiffusion gels using specific antibodies to the other eosinophil basic proteins. Purified neutrophil chymotrypsin-like cationic protein, elastase²⁵, lactoferrin²⁶ and myeloperoxidase²⁷ were also prepared. The binding of antibodies EG1 and EG2 to these purified granule proteins was assessed using microtitre plates (Dynatech) coated with 5 µg ml⁻¹ protein overnight at 4 °C. Unattached sites were blocked with 1% gelatin at 37 °C for 1 h. Endogenous peroxidase activity was removed using 0.3% H₂O₂ in methanol. The amount of antibody bound to these proteins was measured at 450 nm using peroxidase-conjugated rabbit anti-mouse IgG (Miles-Yeda) and H₂O₂ with *o*-phenylenediamine²⁸ (Sigma) and a Titertek multiskan reader (Flow).

* Significant binding above background levels.

titrating them against both cationic proteins (Fig. 1a). Antibody EG2 was studied further in a competitive inhibition assay with purified ECP and EP-X. Antibody EG2 was first incubated with EP-X, then residual binding for ECP was assessed. It was found that increasing amounts of EP-X caused a dose-dependent decrease in the binding of antibody EG2 to ECP (data not shown). In order to confirm the specificities of these two antibodies, western blots were made of eosinophil granule proteins, and the binding of antibodies EG1 and EG2 was assessed (Fig. 1b). These blots showed that antibody EG1 bound only to ECP, whereas antibody EG2 bound to ECP and EP-X.

The antibodies were then used for immunocytochemical staining of blood eosinophils from normal people and patients with the hypereosinophilic syndrome. In this disease, blood eosinophils may be degranulated and contain cytoplasmic vacuoles, which are thought to be areas of granule solubilization and secretion²². Antibody EG1 stained all the eosinophil granules in 25% of normal blood eosinophils (Fig. 2a), and all the granules in 70% of blood eosinophils from patients with the hypereosinophilic syndrome (Fig. 2g). Antibody EG2 did not stain normal blood eosinophils (Fig. 2b) but did stain them when: (1) They had phagocytosed zymosan-C3b particles. In some preparations, staining was found on the surface of adjacent cells, suggesting that the granule proteins had been secreted externally (Fig. 2d) (antibody EG1 also stained phagocytic vacuoles produced by zymosan-C3b, (Fig. 2c). (2) After incubation with eosinophil cytotoxicity enhancing factor⁹ (Fig. 2e, f). Eosinophils which were incubated without this factor were not stained by antibody EG2 (Fig. 2f). (3) Blood eosinophils were obtained from patients with hypereosinophilic syndrome (Fig. 2h).

As EG2 recognizes antigens that are stable in formalin-fixed, paraffin-embedded tissues, we were able to examine the occurrence and distribution of activated and degranulating eosinophils in the skin of patients with chronic urticaria. Large

numbers of activated cells were found in the skin biopsies from 12 out of 14 patients studied, and in some patients this was associated with areas of extracellular deposits of eosinophil secretion products (Fig. 3). Disease severity appeared to correlate with the presence of these activated cells.

The cross-reactivity of EG2 on ECP and on an epitope on EP-X suggested that there were structural similarities between these two proteins, at least on secretion. As these two cationic proteins are of similar molecular size, with closely related physicochemical and biological properties⁶, it is possible that they are structurally related.

The observation of numerous activated eosinophils and their secretion products in the skin supports the view that eosinophils may contribute to tissue injury. It is now possible to extend these studies to other diseases in which eosinophil activation and degranulation in tissues have been thought to occur. The ability of monoclonal antibodies to detect antigenic differences between storage and secreted forms of granule proteins may have many applications in the study of activation and degranulation in secretory cells, in addition to their more obvious use in defining the tissues in which these processes occur.

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Erratum

Determination of the Si-O-Si bond angle distribution in vitreous silica by magic angle spinning NMR

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THE letter with the above title appeared in the 5 April issue (308, 523-525; 1984) with an incorrect initial for one of the authors—E. Dupree should have read R. Dupree.

The trouble with technology

David L. Sills

Normal Accidents: Living with High-Risk Technologies. By Charles Perrow.
Basic Books, New York: 1984. Pp.386. \$21.95.

KARL POPPER once noted that people can be divided between those who see the world as analogous to clocks, in which cause and effect are always mechanically related, and those who see the world as clouds, each particle acting erratically with only the broad shape of things moving in understandable ways. Appealing as this imagery is, however, it will not do to explain the large-scale accidents that occur in a high-technology society: the nuclear accident at Three Mile Island; the dioxin contamination of Seveso; the crash of a large aircraft; the collision of two ships at sea; the failure of a large dam. These accidents are the products of neither clockwork-like processes nor random events. What they are the product of is the theme of this book.

A place to begin is the famous 1966 accident at the Fermi nuclear power plant near Detroit, Michigan — so near, that a popular account of the accident by John Fuller is entitled *We Almost Lost Detroit*. In *Normal Accidents*, Charles Perrow, a professor of sociology at Yale University, gives a technical account of the Fermi accident, an account that points out how instructive the accident was to the designers of nuclear power plants. Perrow remarks that "In our accident is our salvation".

There is a hint of sarcasm in this comment, but there is a larger dose of truth. Accidents have become the X-rays, the litmus paper, the illness, the diagnostic test — whatever metaphor you like — of high-technology systems. Engineers describe how complex machines and machine systems are supposed to work; we find out how they actually work (or fail to work) when there is a serious accident.

It was a major nuclear accident, the one at Three Mile Island in 1979, that turned Perrow's attention to accidents in general. A specialist in the sociology of organizations, he soon learned that events at TMI were not simply the result of an engineering failure or the result of operator error; rather, they were a consequence of systems failure. All of us who pored over the volumes of investigative reports on TMI know how irrelevant it is to blame the accident upon the failure of a relief valve. But what was to blame? In a sense, TMI was a real-life *Rashomon*, each of us seeing in it what our training and predisposition caused us to see. The electrical engineering journal *Spectrum* featured, in its special issue on TMI, a full-page, four-colour diagram of the relief valve; political scientists saw the accident as a regulatory failure; human-factors engineers focused upon the confusion in the control room;

lawyers saw liability; and sociologists concluded that it was a social system that had failed.

What Perrow learned from his research into the accident at TMI is that there was no coherent theory of accidents in either the engineering or the social science literature, so he set out to create one. The result is a well-written, wide-ranging book, full of insight, that may mark the beginning of a science of accident research. Since Perrow is an outsider to all of the many technical fields reviewed in the book, ranging from nuclear power to marine transport to DNA research, experts may challenge his sources and point out his errors. But they will read his book and they will learn from it.

Perrow's central thesis is that accidents are inevitable — that is, they are "normal" — in technologies that have two system characteristics that he terms "interactive complexity" and "tight coupling". Using these concepts, Perrow constructs a theory of systems which he believes to be unique in the literature on accidents and the literature on organizations. His theory concentrates upon the properties of systems themselves, rather than on the errors that owners, designers and operators make in running them. He seeks a more basic explanation than operator error; faulty design or equipment; inadequately trained personnel; or the system is too big, under-financed or mismanaged.

"Linear interactions", according to Perrow, are those interactions of one component in the system with one or more components that precede or follow it in the sequence of production. "Complex interactions" are those in which one component can interact with one or more other components outside the normal production sequence, whether intentional or not; they involve unfamiliar sequences, or unplanned and unexpected sequences, and they are either invisible or not immediately comprehensible.

"Tight coupling" is a term borrowed

Type of coupling	Type of interaction	
	Linear	Complex
Tight	Dams	Nuclear power
	Marine and rail transport	Nuclear weapons accidents
		DNA research
		(1) (2)
		(3) (4)
Loose	Assembly-line production	Mining
	Most manufacturing	

from mechanical engineering. It means that two elements are joined in a way in which there is no slack or buffer or give between them; what happens in one element directly affects what happens in the other. "Loose coupling" is of course the opposite. These dimensions can be cross-tabulated, giving four cells. A simplified version of Perrow's basic scheme is shown at the foot of the preceding column.

Building upon this scheme, Perrow introduces such concepts as catastrophic potential and the costs of alternatives. This makes it possible for him to draw conclusions and make policy recommendations based upon how a technology scores on all of these ratings.

His basic finding is that nuclear weapons and nuclear power have the worst scores of all; his recommendation is to abandon them. Marine transport and DNA research score in the middle; he recommends that their use should be restricted. Space travel, dams, mining, chemical plants and air travel are left to be tolerated and improved.

I have tried to convey something of the impressive theory that leads to these conclusions, and have hardly noted the wealth of fascinating sociotechnical detail that constitutes the bulk of the book. The chapter on marine transport is particularly well done; it will certainly frighten all weekend sailors who venture out to the edges of a watery chaos where currently more than one large ship a day is lost at sea. "Elites", Perrow notes, "do not sail on Liberian tankers" (p.173).

As a sociologist, I applaud Perrow's ideas of complex interaction and tight coupling; these are new social concepts that are clearly essential for the analysis of technological systems. But in going so far down the policy road as to recommend that nuclear power should be abandoned, doesn't Perrow become a latter-day Luddite, using the tools of science to smash a technology he does not trust?

Nuclear power in the United States may not survive its current economic and regulatory troubles, but discussion continues. Only a small part of the debate concerns plant safety: economic competitiveness, nuclear arms proliferation and nuclear waste disposal are the salient themes. Outside the United States nuclear power is doing rather better, and the *New York Times* reports that General Electric, Westinghouse and Combustion Engineering are currently seeking to sell China some \$4 to \$5 billion dollars worth of equipment that they cannot sell domestically. There seems to be life in the old dog yet.

The difficulty may be in Perrow's yearning for significance, his dissatisfaction with merely creating a new science of accident research. His book will markedly improve discourse on the nuclear power debate; it should not have attempted to decide it. □

David L. Sills is the Executive Associate of the Social Science Research Council, New York.

More and more about less and less

V.E. Cosslett

Transmission Electron Microscopy: Physics of Image Formation and Microanalysis.

By Ludwig Reimer.
Springer-Verlag: 1984. Pp.521.
DM 118, \$45.80.

IT IS over 50 years since the electron microscope was invented in Germany and nearly 45 years since the first comprehensive book about it appeared (M. von Ardenne's *Elektronen-Übermikroskopie*, published by Springer-Verlag in 1940). Fortunate though we are to have the founders still with us, they have developed in rather different directions: the originator of the instrument (E. Ruska) towards the attainment of high resolution and von Ardenne into biological problems.

In the interim there has been an increasing number of treatments of the subject, from review articles to multi-authored series. Desirable as a uniform coverage by a single author may be, it is hardly practicable any longer except at an elementary level. The last attempt at a full treatment known to me also came (appropriately enough) from Germany,

and indeed from the same publishers — *Elektronenmikroskopische Untersuchungs-und Präparations — Methoden* by L. Reimer. The growth of the subject is mirrored in the size (598 pages and 247 illustrations) of the second edition (1967), as compared with the original 300 pages and 135 illustrations in 1959.

The need for an even greater expansion is acknowledged by Professor Reimer in the preface to his new book. To meet such a daunting task he now confines his treatment to the theoretical basis of electron beam imaging and even more narrowly to that in transmission electron microscopy, leaving the large field of scanning almost entirely to one side.

After a short introductory chapter, the physical groundwork is set out: the particle optics and the wave optics of electron beams in two separate chapters, including lens aberrations in the former and diffraction in the latter. The functions of the several parts of a transmission electron microscope are then dealt with in turn, from the electron source to the camera and alternative detecting systems. Beam-specimen interactions, including scattering, energy loss and the origin of phase contrast occupy two chapters (100 pages), with the two following (130 pages) devoted to the details of diffraction theory, the formation of diffraction contrast and crystal structure imaging. These four chapters comprise the heart of the book. Chapter 9 is concerned with various aspects of analytical electron microscopy and the last chapter with the vital problem of specimen damage caused by the electron beam.

The subject of transmission electron microscopy is thus covered by the author in all its basic instrumental aspects, but with only a few incidental asides about practical applications. These were described at length in his 1967 book, half of which was devoted to an account of methods of specimen preparation. More recent developments can be followed up in the many congress proceedings since published.

Reimer's new text ends with a formidable list of references, arranged by the chapters in which they arise and running to over 150 pages in all. As a whole, the book gives an authoritative and up-to-date account of the basic principles of a subject of ever-increasing scientific and technological importance, clearly presented and intelligible to anyone at post-graduate level with a good grounding in mathematics. Some acquaintance with the Schrödinger equation and with diffraction theory would be advantageous but not essential to an understanding of the physical ideas. As stated in the preface, the treatment evolved from a series of university lectures and so it would be a good basis for any similar course. □

V. E. Cosslett is Emeritus Reader in Electron Physics at the University of Cambridge.

Rights and wrongs

T.L.S. Sprigge

The Case for Animal Rights.

By Tom Regan.
Routledge & Kegan Paul/University of California Press: 1984. Pp.425.
£17.95, \$24.95.

ANIMAL rights are proving an active subject for philosophers — this work is only the most recent of several books arguing that the ethical principles which govern relations between human beings imply that man does wrong to treat animals as he does, in the kind of animal farming, scientific research and blood sports sanctioned by our current laws and mores. Such writers have helped to change the image of the animal rights (or liberation or welfare) movement. Whereas it was once the habit of animal experimenters or factory farmers to dismiss the opposition as "dear old ladies" and "animal lovers", only the exceptionally blinkered do so now. For these philosophers have insisted that they are not necessarily sentimental about animals; rather they are simply concerned to point out in a cool, rational way that we are behaving wrongly, judged by our own most reasoned standards, and that we are morally obliged to mend our ways.

There have also been those who have defended the principles underlying current practices. Philosophers on each side have typically started with one of the main ethical theories advanced for intra-human relations, and then applied their favoured theory to human behaviour towards animals. Four such theories have figured prominently in the discussion: utilitarianism; contract theories; perfectionist theories; and rights-based theories. This last is the viewpoint defended by Professor Regan.

Utilitarians typically contend that the basic factors to be considered are good experiences promoted and bad experiences prevented, these usually being equated with pleasures and sufferings. The implication is that so far as animals suffer and enjoy life as genuinely as we do, their experiences must be taken into the reckoning on a par with ours. Many utilitarians have concluded that most animal suffering arising from these practices in anything like their present form is not justified, since either the good done (a new drug, say) fails to outweigh the suffering, or equivalent good could be achieved more cheaply in terms of suffering. The main — and very effective — exponent of this view is Peter Singer.

Objections to this line have come not only from non-utilitarians but from other utilitarians, the most usual defences of animal research and modern farming applying a utilitarian ethic. To me they seem ineffective, however, since they rest partly on a failure to take animal sentience

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sufficiently seriously, partly on unimaginativeness regarding the possibilities of change in human-life-style. With so many under-funded fields in which welfare and knowledge could be increased, why not favour those employing methods not harmful to sentient creatures?

Contract theory sees moral principles as rules of the game of life which morally-conscious agents implicitly do, or if rational would, agree to abide by in their mutual relations. Since animals cannot be party to such a contract, and thus do not belong to the moral community so created, contract theorists give at best a secondary place to human duties towards animals.

A more formidable counter to the utilitarian attack on current practices comes from "perfectionist" ethics; here sentient beings are ranked as different one from another in inherent value. Such a theory is readily used to justify the human exploitation of animals as simply means to the promotion of values only realizable in human life.

Professor Regan contends that each of the first three theories is to be rejected independently of its implications for the animal issue. He is surely right that the contract theory is totally inadequate. But his criticism of perfectionism — a stance of crucial importance — seems rather slight, turning simply on the contention that it implies an intolerable value-ranking of human beings and thus justification for the making of inferior human beings means to the good of superior ones.

Regan's most distinctive line, however, as a proponent of animal rights, lies in the argument (with which I wholly disagree) that utilitarianism cannot provide the basis for the condemnation of current treatment of animals which Singer erects upon it. Simply in terms of the aggregate of pleasure gained and pain prevented, present practices may be largely justifiable, he says. In contrast, they are totally wrong when viewed in the light of an adequate rights-based theory.

According to rights theory, there are valid moral principles such as that every individual possessed of a certain inherent value has the right not to be harmed, to prevent less serious harms to others (however many of them there may be) or even more serious harm, where this is not one they have some special right to be

protected from (as we do not have the right to be protected from sickness at another's cost in life and health). Regan's great challenge to conventional morality is that he argues that this inherent value is shared by every individual with certain mental capacities — such as an ability to plan — and that at least all mammals possess such capacities as truly as we do. No such individual should be regarded as a renewable resource for the purposes of others, nor even merely as one of a set of receptacles for as much aggregate pleasure and as little pain as possible, as we all are for utilitarianism.

Regan's book poses a carefully worked out challenge to all those who warm to an ethic such as his when *only* human beings are being considered. Such people should ponder whether he may not be right that at least all mammals can be denied such value, and consequent rights, only through arbitrary restrictions made in favour of our own species. They will probably find in Regan's book replies to the more obvious objections which occur to them.

Regan's work, however, does have certain limitations. Great emphasis is laid on reflective intuitions on moral matters without its being made clear on what we are to reflect in arriving at them. His discussion of animal consciousness properly opposes extreme views which would deny it altogether, but says little as to how we might improve our knowledge of it. Moreover, he muddies the issue by resting his case for animal consciousness on the need for it to explain behaviour, whereas even if physicalistic explanations which bypass it are possible it is still as much there, as in human beings, as something ethically significant. Finally the rather mystical notion of an inherent value present in every man and mammal, and perhaps beyond, is insufficiently explained, while the claim that it is equal in every case is weakly grounded on the impossibility of measurement and unacceptable elitism of its denial. For all that, this is a telling attack on present human attitudes and behaviour towards animals, one which is based on views implicit in widely professed ideals. □

T.L.S. Sprigge is Professor of Logic and Metaphysics at the University of Edinburgh.

Pieces of science

John Maddox

Man Suddenly Sees to the Edge of the Universe.

By Richard Casement.

The Economist, London/Open Court Publishing, Box 599, La Salle, Illinois: 1984. Pp.204. £4.95, \$12.95.

The Economist, increasingly the thinking man's *Time* magazine but with a British accent, has been so moved by the untimely death two years ago of its science writer, Richard Casement, that it has now issued a collection of his published pieces under a characteristic title. The book is a good read but also splendid illustration of the symbiotic interaction between writers and the papers for which they work.

Casement was not by training a scientist in the strict sense, but one in whom education (philosophy, Oxford) and early experience as a journalist (the *London Times*) had cultivated a vivid curiosity about science and technology in relation to the world at large. Casement's most tangible achievement was to persuade *The Economist* to give him an outlet — a section in each issue of the weekly magazine. But his distinctive style may wear even better.

There is a splendid piece (reprinted here) about the sociology of the Cavendish Laboratory, raising in 1982 the question that now more urgently bothers the people at Cambridge — will this great laboratory manage to stay in the forefront of physics, and how? The anthology shows that Casement also considered that readers of *The Economist* should be told about evolution, neurophysiology and psychiatry — and he was right. But how can one man, without formal training in such subjects, write confidently about all of these matters? Casement's trick was to talk endlessly to people with interesting things to say. Now and again, this anthology shows, he was taken in, but mostly he seems to have emerged with a better judgement than his informants of where their work would lead.

There is one debt that Casement owed to *The Economist*: the belief that journalism practised by the application of intelligent and untrammelled curiosity to complex problems is one of the few remaining honourable professions. Casement's style was plainly also influenced by his fellow journalists on the magazine, with their two-word sentences and three-word epigrams. His strength was that the need for compression forced him into generalizations that were almost always true.

The Economist is understandably shocked to have lost this talent, and generous to have collected Casement's pieces together in this way. □

John Maddox is Editor of Nature.



The anti-vivisection lobby — moral support from philosophers?

Nature pictured

Charles A. Fleming

Sydney Parkinson: Artist of Cook's Endeavour Voyage.

Edited by D.J. Carr.

Nova Pacifica, Wellington/Australian National University Press, Canberra/Croom Helm, London: 1984. Pp.300. NZ\$85, A\$49.95, £29.95.

BICENTENNIAL commemorations in 1969, and the Hakluyt editions of Cook's Voyages (edited by J.C. Beaglehole, and published between 1955 and 1967), stimulated still further publication of pictures and descriptions that constitute the historical treasures of the lands James Cook made known to the Western world. To dwellers in the "new" countries of the Southern Hemisphere, these first European observations of our homelands are precious cultural resources, basic to understanding our history and the environments we have modified so severely.

Sydney Parkinson, whose life, sketches and paintings are the subject of this book, was already skilled as a nature artist when he joined Joseph Banks's team in 1767 at the age of 22. In the four remaining years of his life, Parkinson illustrated most of the natural curiosities collected on the voyage of the *Endeavour*, and also drew coastal landscapes for the Master's log. In New

Zealand and Australia, the harvest of novelties was so rich that priorities were set; botany got preference over zoology, and Parkinson skilfully sketched pencil outlines with representative details coloured in, a shorthand technique that enabled artists in London to finish many of the plant pictures.

In his preface, D.J. Carr acknowledges the encouragement of the late Averil Lysaght, "clearly the writer of choice for a biography of Parkinson". Between them, the editor and Wilfrid Blunt cover Parkinson's life story very well, however, and Blunt also describes the *Endeavour* voyage using his text from *Captain Cook's Florilegium* (1973). Naturalist readers may jib at his *ex cathedra* judgement of Spöring's "pedestrian" sketches and of his suspected use of a ruler (*horreur!*), while Blunt perhaps also underestimates the increasing interest in natural history during the past half-century in dismissing invertebrate pictures as of little interest to the general public.

Chapters 3 to 7 deal with plants of Madeira and South America (Edwards), Tahiti (Fosberg and Sachet), New Zealand (Godley), Australia (Henderson) and Java (Stearn). Each of these accounts consists of a double-column narrative text, in which illustrations are interspersed with extended single-column captions in smaller font, an at times somewhat confusing format. Chapters vary in style, Henderson giving etymology of Linnean names while other authors devote more space to history of nomenclature, biology or biogeography.

In his contribution on Parkinson's animal paintings, Alwyne Wheeler throws new light on the work of Banks's research team and offers explanations for their puzzling neglect of land animals in favour of plants. Wheeler also includes an account (and reproductions) of Parkinson's early copies of paintings of Ceylon animals (J.G. Loten coll.), some of which were later published by Pennant and by Forster. The paintings for Banks in 1767-1768 are not sampled, however, being already covered in Lysaght's *Joseph Banks in Newfoundland and Labrador 1766*. Parkinson's "meticulous accuracy and loving detail" in depicting insects and minute crustaceans from the plankton contrast markedly with his perfunctory sketches of kangaroos and other novel land vertebrates. Fishes dominate the 39 vertebrate drawings included here, most of which are beautiful colour reproductions though some are over-reduced and framed by too much blank paper.

The editor adds appendices on the identity of Cook's kangaroo(s), and on birds in Parkinson's sketchbook that were his own versions of species illustrated by George Edwards, in a work probably from the *Endeavour's* library. This links up with a short chapter on Malayan boats by G.A. Horridge, who identifies two folios from the sketchbook, showing outrigger boats with triangular sails, as Parkinson's

renderings of the famous Marianas "flying proa", a style of vessel he never saw, already on the decline 40 years before Cook's voyage. Parkinson studied a diagram in a copy of Anson's *Voyage* (1748), saw it sailing "in his mind's eye" and drew perspective sketches.

The final chapter, "Coastal Profiles and Landscapes" by J.R.H. Spencer, is of special interest to New Zealanders. Spencer



Purple-rumped sunbird (*Nectarinia zeylonica*), depicted by Sydney Parkinson, 1767.

compares Parkinson's sketches and romantic pen-and-wash drawings with Spöring's drawings and modern photographs of the Maori fortified *paa* at Motuarohia, Bay of Islands, supported by a site plan from his own surveys in 1974. He demonstrates how Parkinson, minutely accurate in depicting coasts and scientific specimens, rearranged or supplemented the actual scene "to provide the elements of a pleasing composition".

Welcome as it is, the book has some annoying defects. How long must bibliophiles tolerate square books that fit no conventional bookshelf? On the dust cover, one of Parkinson's finest pictures is marred by (and obscures) the superposed lettering. (But, on the other hand, the endpapers are beautifully conceived and executed.) And while the much-reduced plates are exquisite, the yellowish-cream backgrounds (absent from *Florilegium* plates) sometimes obscure Parkinson's pencil outlines of details.

In each of the lands the *Endeavour* visited, naturalists without access to Banks's *Florilegium* will want complete coverage of that land's pictures; future demand is likely to be for separate works containing all of his drawings from each country in turn. Meanwhile, this volume gives overdue tribute to Sydney Parkinson and offers a wide selection of his work in one volume. □

Sir Charles Fleming is Honorary Lecturer in Geology at the Victoria University of Wellington. From 1953 to 1977 he was Chief Palaeontologist to the New Zealand Geological Survey.

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RNA probes prepared *in vitro*

from P. F. R. Little

A new technique of synthesizing single-stranded RNA from DNA vectors promises much.

ADVANCES in molecular biology over the past decade have been due in large measure to the development of necessary techniques. Central to the technology is the ability to radiolabel DNA *in vitro* to high specific activity. Two methods are currently used to generate DNA molecules of high specific activity — labelling of double-strand DNA by 'nick-translation' and labelling by DNA synthesis primed off a single-stranded DNA template. Now a new technique that allows the synthesis of single-stranded RNA from specially constructed plasmid DNA vectors may lead to interesting new developments.

Some bacteriophage, for example the T7 coliphages, encode RNA polymerases that only initiate transcription from their own phage promoters. However, Butler and Chamberlin¹ identified an RNA polymerase encoded by a bacteriophage, SP6, of *Salmonella typhimurium*, which, unlike the equivalent T7 enzyme, is easy to purify, very stable and highly active *in vitro*. SP6 RNA polymerase is a single polypeptide chain of molecular weight 96,000 and is highly specific for SP6 promoters. When Butler was in Maniatis' laboratory at CalTech, ideas began to emerge as to how the polymerase could be exploited to produce transcripts from genes cloned into special vectors. These ideas are illustrated in Fig. 1. A single SP6 promoter cloned onto pBR322 was shown to be transcribed very efficiently *in vitro* (Fig. 1a). Any DNA fragment inserted downstream of the SP6 promoter (Fig. 1b) was also transcribed, producing an RNA copy of the inserted DNA. Restriction enzyme cleavage of the DNA template downstream of the inserted gene provides a discrete terminus for the RNA (R in Fig. 1c). Thus an RNA fragment of precise length (a 'run-off transcript') could be produced from any DNA cloned into the vector (Fig. 1c, d). The length is defined by the precise relationship of the promoter and DNA inserted and by the 'run-off' cleavage site and the transcript is either mRNA-like or 'anti' mRNA depending on the orientation of the gene or DNA within the plasmid. The specificity and asymmetric transcription of the SP6 polymerase and its promoter in this system is in contrast to *Escherichia coli* RNA polymerase and strong promoters such as *lac* in similar constructs. The *E. coli* enzyme initiates at sites other than *lacP* and extensive isolation of either DNA or RNA fragments is required (my unpublished data).

The first of a series of vectors designed to permit the transcription of any piece of DNA was constructed by Butler and Little

(see ref. 2) by insertion of a series of restriction sites, present only once within the plasmid, downstream of the SP6 promoter. This vector was then used by M.R. Green, D.A. Melton, T. Maniatis and co-workers at Harvard University (in preparation), and L.M. and R.C. Angerer and co-workers at the University of Rochester (personal communication), to generate a series of increasingly refined plasmids with differing restriction sites downstream of the SP6 promoter.

The SP6 polymerase vector system has so far been exploited in three different ways. Green *et al.*² cloned the human β -globin gene into an SP6 promoter vector and used the polymerase to synthesize substantial quantities of a 'primary transcript' of the globin gene, containing all its introns, and then showed that the *Xenopus* oocyte could accurately splice this RNA.

Kraimer *et al.*³ have extended these

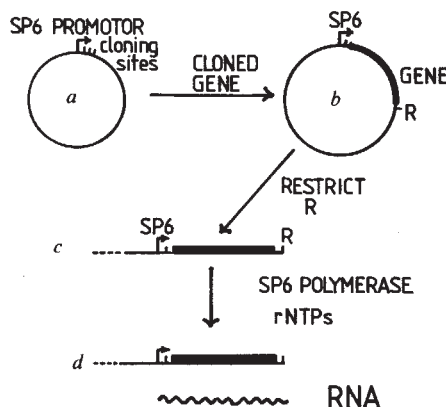


Fig. 1 Use of SP6 RNA polymerase in producing RNA probes *in vitro*.

studies to human cell extracts. The availability of unlimited amounts of precursor RNA allowed optimization of the splicing system and splicing efficiencies of 80–90% were achieved. Similar experiments have demonstrated 3' processing of histone gene transcripts⁴.

Zinn *et al.*⁵ developed the use of short SP6 transcripts of interferon (IFN) genes to quantitate IFN RNAs in a variety of experiments. They described a technique known as 'RNase protection' to do this. A highly radiolabelled RNA of defined length complementary to part of IFN mRNA is synthesized by SP6 polymerase. The radiolabelled 'probe' RNA is then hybridized in excess to the cell RNA and forms a double-stranded RNA-RNA hybrid. The SP6 transcript will contain stretches of RNA not homologous to IFN mRNA — transcripts from the SP6 DNA and plasmid sequences, and DNA not

transcribed into mature IFN mRNA. These single-strand regions can be 'trimmed' away by a mixture of RNase A and T₁ and an RNA of characteristic size will be protected by virtue of its double strandedness. The size and yield of this RNA is measured by acrylamide gel electrophoresis and autoradiography. Since the precise structure of the SP6 transcript is known, conclusions can be drawn as to sites of initiation of IFN mRNA synthesis, giving precise quantitation of IFN mRNA levels in cells. Splicing patterns can be analysed in a similar fashion. As little as 0.1 pg of RNA can be detected by this procedure (ref. 5 and D. A. Melton, P. A. Krieg and M. R. Green, personal communication), and experimental conditions of hybridization and RNase digestion are not as critical as in end-labelled S₁ analysis. RNase protection is probably 10–100 times more sensitive than the latter technique.

The polymerase has also been used to generate single-strand probes of the highest specific activity for use in Northern and Southern blot hybridizations (K. Zinn *et al.*, personal communication). Church and Gilbert⁶ have developed a method for directly sequencing genomic DNA by Southern blotting (genomic sequencing) and highly radiolabelled probes generated by SP6 were critical in this development. Cox *et al.*⁷ have used SP6 generated probes for *in situ* hybridization. Signal and signal to noise levels are superior to either nick-translated or single-stranded DNA probes.

SP6 RNA polymerase is commercially produced and vector plasmids are freely available. Nick-translation will probably remain the method of choice for labelling DNA fragments isolated by gel electrophoresis for example, but the high specific activity of RNA probes and the low background after RNase treatment will be increasingly exploited. The necessity of cloning DNA sequences into special vectors will in part be alleviated by incorporating SP6 promoters into, for example, the widely used Messing pUC series of plasmids. □

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New equipment for biotechnology

The Biotech 84 exhibition takes place in London's Wembley Conference Centre on 15-17 May. The following pages describe some of the new products on view.

● Techne, manufacturer of temperature controlled laboratory equipment, is displaying two main product lines at Biotech — biological stirrers and a new range of thermostatically controlled water baths. The MCS biological stirrer, of particular use in cell culture, features a controlled stirring action with "Softstart" capability to avoid excessive turbulence within the medium and so eliminate cell damage. The MCS can either be stirred continuously or at predetermined intervals: this can improve cell yields particularly during the attachment stage. Five redesigned thermoregulators are included in the new Techne range. The simplest model, the TE-8J Tempette Junior, is an analogue version with good precision of plus or minus 0.05°C. At the top end of the range is the digital TU-2D Tempunit with plus or minus 0.005°C precision, remote control and programming facilities.

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● A representative selection of Beckman laboratory equipment is on show at Biotech. Areas covered include centrifugation, represented by the latest L8M ultracentrifuge which operates at speeds up to 80,000 r.p.m., and the J2-21M high-speed centrifuge with speeds up to 21,000 r.p.m. Spectroscopy products will include the DU-7 UV/visible spectrophotometer with slab gel accessory, and there will be one of the latest bench-top liquid scintillation counters on show. Instrumentation will also be demonstrated for use in DNA and protein synthesis, amino acid analysis and HPLC techniques.

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● The Swiss company Bioengineering produces a full range of fermentation equipment. Cell culture can be carried out at a variety of capacities ranging from the bench-top "Baby" to full research and production pilot plant scale. Complete packages are available for bacterial, fungal, plant or animal cell culture at volumes of from 1 to 5,000 litres. Even larger capacities can be supplied to special order — vessels of 30,000 litres have been produced. Also on the stand at Biotech will be Bioengineering's data logging and control package based on Hewlett-Packard hardware, and a new instrumentation package based on a modular concept. The instrumentation for each parameter to be measured or controlled is contained in an individual module, and each module is capable of either rack or free-standing mounting.

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● The Arnold R. Horwell display at Biotech features products from Belco Biotechnology. The large roll-in incubator can accommodate the Belco cell production roller apparatus and eight decks; if fewer decks are fitted, then shelves may be used in the upper half to accommodate rocker platforms, magnetic stirrers or other necessary equipment. Other Belco equipment on view will be rocket platforms, controlled atmosphere culture chambers and magnetic stirrer platforms. Incubators on display will be the water-jacketed CO₂ model and the bench-top incubator including cell production roller apparatus. The Horwell stand will be completed by a display of Sterilin tissue culture ware, Hettich centrifuges and the Tecnomara Pipet-Boy liquid handling device.

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● Schaefer Instruments of Wantage, Oxfordshire, will be showing the Model MGA-1200 Perkin-Elmer process mass spectrometer with a microprocessor-based controller for automatic calibration and other essential functions. This instrument's drift stability makes it suitable for on-line fermentation control. The stand will also feature representative examples of Datametrics high precision Barocel capacitance manometers and a gas mixing system based on these, and the new Series 800 flow controllers.

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● Biotech sees the launch of new products from Pharmacia Fine Chemicals for industrial scale chromatography including a new range of cost-effective columns. Process systems for the downstream purification of biological products will also be on show. Personnel from Pharmacia Fine Chemicals will be available on the stand to discuss protein purification and electrophoresis and provide information on the newly announced range of biochemicals and molecular biology reagents from Pharmacia P-L.

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● The latest addition to the Cruachem DNA synthesis product range is being launched at Biotech Europe. This is a very simple, manually-operated DNA synthesizer, designed by Cruachem Inc. in the United States. This simple module contains everything needed to perform DNA synthesis and is intended mainly for laboratories with an occasional need for defined-sequence DNA molecules as a cost-effective alternative to custom DNA synthesis.

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DNA synthesis by Cruachem (top) and Bioengineering's small bench-top fermenter.

● The Queue range of ultra low-temperature freezers, CO₂ incubators and shakers can be seen on the Camlab stand. Cryostar freezers are single compressor, ultra-low temperature, biological storage freezers which guarantee the temperature performance in +35°C ambient. The range includes -90°C upright models and -75°C, -100°C and -135°C chest models. The single compressor refrigeration system eliminates the problems encountered with the multiple compressor cascade designs. Queue CO₂ incubators for cell culture do not utilize sensors and the gas levels are not affected by changes in chamber temperature or relative humidity. The incubators are of stainless steel construction with continuous fresh air exchange and CO₂ concentration is maintained without calibration. The new orbital shaker is a low-profile unit which is available as an independent unit for use in incubators, environmental chambers or on the laboratory bench, or as a combination incubator/shaker, designed to deliver precise orbital motion in a controlled temperature environment.

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● The Centre for Applied Microbiology and Research (CAMR) at Porton Down is involved in the application of biotechnological expertise to the development of new and improved products, methods and equipment which relate largely, but not exclusively to the field of health care. CAMR, part of the Public Health Laboratory Service, has a particular interest in collaborative relationships with commercial, industrial, governmental and other organizations. Examples of such collaborative work will be on display at the CAMR stand, as will literature giving information about the broad range of facilities and knowledge which is available at Porton, including news on the new facilities to be opened shortly. A major stand exhibit will be an active on-line computer link to the CAMR pilot plant at Porton.

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● Anderman and Co., UK distributors of Schleicher and Schuell products, will be exhibiting a wide range of membranes and associated equipment for the genetics market including nitrocellulose filters, cellulose acetate membranes, diazotized paper and other filtration media. Amongst the equipment being displayed will be two, four and ten place vacuum filter holders for binding, hybridization and transfer studies, the SRC 96 micro-sample filtration manifold and Elutip-D columns, a rapid inexpensive method of purifying and concentrating DNA.

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● At Biotech the Boehringer Mannheim stand will be providing information on a comprehensive range of restriction enzymes, DNA and RNA modifying enzymes, linkers and auxiliary reagents. Among the enzymes available are *ApyI* and *DpnI*, both useful in studying the role of methylation. Enzymes for special industrial applications include immobilized penicillin-G amidase, α -galactosidase, aminoacylase, fumarase, carboxypeptidase, catalase and β -galactosidase. For the analysis of raw materials in process control and quality assurance, Boehringer Mannheim markets a number of enzymatic analytical kits.

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● The new Acroflux filtration system from Gelman Sciences is a cross-flow filtration system in an inexpensive, convenient, easy-to-use unit capable of handling anything from a few litres of small batch concentrates to high-volume process requirements. For applications such as cell harvesting, solution concentration or general clarification, Acroflux offers a longer filter life than conventional systems together with backflush and a re-use facility at a reasonable cost. Details of Gelman's other specialized products are contained in the publication "Innovations in Filtration" which covers a wide range of disposable filter devices for biotechnology applications.

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The Sartorius MP8 1900 balance.

● New from Amicon is a 0.1 μ m anisotropic hollow fibre cartridge for the downstream processing of fermentation broths and yeasts. When used for cell harvesting of *Escherichia coli* (to 100 gl^{-1}), typical filtration rates of 150 $\text{l h}^{-1} \text{m}^{-2}$ are achieved with yields greater than 98% and cell densities over 90% by volume. The systems completely contain the process fluid with no aerosol contamination. The cartridges are simple to regenerate and scale up is achieved in modular form. Also from Amicon, the Vitafibre II hollow fibre cell culture system is suitable for long-term production of many monoclonal antibodies, growth factors, plasminogen activators, interferons, viruses and tumour associated antigens.

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● The Sartorius range on display at Biotech covers precision weighing, laboratory filtration, shaking, mixing, stirring and homogenizing. The latest MP8 series of electronic balances includes models suitable for use at the microgram level and others capable of handling up to 60 kg. Filtration systems shown include syringe filter holders, nutrient pad sets, culture media and membrane strips for electrophoresis.

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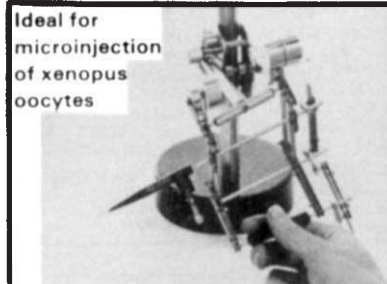
● A full range of laboratory, pilot scale and industrial fermenters, and process control instrumentation is described in the new catalogue from New Brunswick Scientific Co. Included is a wide range of equipment from bench-top fermenters to industrial pilot plant facilities, including micro-processor controlled instrumentation. The fermentation equipment listed ranges from 1 to 2,000 litres in volume, although larger capacities are available when required. New Brunswick's instrumentation and accessories includes digital and analog controllers for accurate control of process parameters, sensors, centrifuges, sterilizers, air incinerators and refrigeration equipment.

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● The P & S Biochemicals stand will be displaying a wide range of restriction and DNA/RNA modifying enzymes, including at least three new specificities. The company's work in identifying and producing new restriction enzymes will be shown and many of its development group activities will be included (in clinical and/or industrial enzyme manufacture). Also on display will be specialty products from Promega Biotec for whom P & S is main UK distributor. This range will include Packagene, Gammarep, RNasin, and a new SP6-derived, high fidelity transcription system — Riboprobe — for the analysis of gene structure and function.

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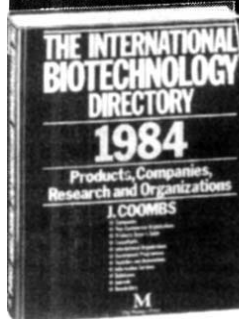
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● The Bio-Rad stand will feature a new modular gradient system for HPLC. Based on the Apple computer, the system produces binary gradients for reverse phase, ion-exchange or hydroxyapatite HPLC. The Model 1305A ultraviolet monitor and analytical refractometer provides sensitive, high performance detection systems for gradient, isocratic or preparative HPLC. Bio-Rad offers a selection of columns for dedicated chemical analysis of organic acid, organic base, protein/peptide, amino acid, nucleic acid, carbohydrate and alcohol. Of particular interest to protein biochemists will be the new "phenyl column" for hydrophobic chromatography in non-denaturing solvents. Latest additions to Biorad's range of electrophoresis equipment include a wide mini sub-cell, allowing rapid screening of multiple DNA samples; a Trans-blot system for the electrophoretic transfer of proteins and nucleic acids and the Model 1800 sequencing cell that gives improved resolution and eliminates "smiling" in DNA sequencing. Other items on the stand will include enzyme conjugated and non-conjugated affinity purified antibodies for antigen detection by EIA, and the new Bio-Dot microfiltration apparatus which has many applications in the fields of DNA, RNA and protein blotting.

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● At Biotech Whatman is showing the latest range of large-scale chromatography equipment and media for downstream processing. This will include the Magnum series 40, 70 and the new 180 production liquid chromatograph column, developed specifically for commercial applications. The Prep-25 column for pilot plant or pilot scale protein separation will also be on show. This column can be filled and eluted easily in one working day and features a specially profiled head ensuring laminar flow and high efficiency. The squat profile of the column allows direct scale-up from the laboratory method without changing chromatographic parameters. The full range of advanced ion-exchange celluloses will be on display including a cell debris remover (CDR) which removes unwanted physical debris and chemical contaminants from fermentation broths and tissue lysates.

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● The Sterilin stand will feature the Belco range of glassware for biotechnology applications. Belco specializes in products such as roller bottles, shaker flasks, micro-carrier systems, rocker apparatus and magnetic stirrers. Belco also supplies a wide range of general laboratory glassware including pipettes with break resistant, hardened tips, and can also manufacture glassware to customer's requirements.

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These notes are based on information provided by the manufacturers. For further details circle the appropriate numbers on the Reader Service Card bound inside the journal.



Belco's range of glassware.

● Biotech Instruments, based in Hertford, represents a number of international companies. Biotronik GmbH, based in Munich, manufactures amino acid analysers. The LC5000 analyser, the latest addition to the range, is a microprocessor-controlled instrument which utilizes the latest column and resin technology to produce fast, efficient and reproducible separations of both protein hydrolysates and physiological fluids. Also on the stand will be products from Applied Biosystems Inc., including the Model 470A gas-phase protein sequencer, capable of routinely sequencing peptides and proteins at levels less than 100 pmol. The Applied Biosystems Model 380A DNA synthesizer is a fully automatic instrument which uses solid-phase phosphoramidite chemistry to produce high purity oligomers. Many users are routinely producing DNA strands up to 60 bases long. Another new product from Applied Biosystems is the 430S peptide synthesizer, a microprocessor controlled, fully automated self-contained system for solid-phase synthesis of peptides using single couplings for most amino acids.

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● New filters from Domnick Hunter for liquids and sterilization will be on show at Biotech. A complete new range of membrane filters manufactured from mixed esters of cellulose, nylon and PTFE is available in disk and cartridge form. Of particular interest is the Asypor highly asymmetrical pore size distribution membrane. It offers double the flow rates and double the throughputs obtained with conventional membrane filters. Traditional chemical and mechanical separations are often too harsh for application in biotechnology. The new crossflow system offers the benefits of high flux rates, high slurry concentrations, gentle operation and the ability to operate under aseptic contained conditions. The release of contaminated exhaust gases into the atmosphere from recombinant DNA fermentation processes is causing concern. Domnick Hunter has available an off-gas filtration system, which cost effectively sterilizes the exhaust gas. The Validator, a complete *in situ* integrity air filter test system is also available from Domnick Hunter. It is a simple, practical and effective portable filter efficiency testing system.

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